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14. ABSTRACT:
Legumain is a recently discovered and only known asparaginyl endopeptidase that is well conserved throughout the biologic kingdoms. We have demonstrated that legumain is highly and inappropriately expressed in 100% human breast cancer specimens as well as murine breast cancer models. We demonstrated that an inactive prototype doxorubicin derived prodrug incorporating a succinyl blocked substrate peptide removable by legumain was effectively activated and tumoricidal in human breast cancer models. We designated this prodrug legubicin. Legubicin is not cytotoxic until activated by legumain due to reduced ability to enter cells and blocked binding to DNA. These properties led to increased tumor exposure and much reduced drug accumulation in normal tissues when administered in vivo. It has markedly reduced cardiac and myelosuppressive toxicities compare to doxorubicin. In this grant application we propose to further develop this prodrug strategy as a potential treatment for breast cancer.

15. SUBJECT TERMS
Legumain, protease, asparaginyl endopeptidase, legubicin, doxorubicin, prodrug, chemotherapy, carcinoma, breast cancer, metalloproteinase, metastasis.

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Legubicin a Tumor-activated Prodrug for Breast Cancer Therapy

Background

Using positional gene expression profiling and high density tissue arrays, we have discovered that legumain is highly expressed by a majority of solid tumors, including 100% breast carcinomas examined [1]. Immunohistochemical analysis of tumor tissues reveals that legumain expressed by tumor cells as well as endothelial cells and tumor associated macrophages (TAM), cells constitute tumor microenvironment. In tumor microenvironment, legumain is present on cell surface in the tumor microenvironment and functional due to the acidic condition existed there. Legumain is a recently identified protease, a distinct member of the C13 family of cysteine proteases [2]. It is well conserved throughout the biologic kingdoms, found first in plants, subsequently in parasites, as well as mammals as an endopeptidase. Legumain is active in acidic pH condition and quickly inactivated under neutral pH. It has a very restricted specificity requiring an asparagine at the P1 site of substrates. Its novel specificity supports that it may be implicated in limited proteolysis consistent with limited proteolytic activation of protease zymogens, as well as selected proteins and peptides. The human legumain gene encodes a preproprotein of 433 amino acids. Mouse legumain shares 83% homology with the human protein [3]. Cells expressing legumain have enhanced migratory and invasive properties. A correlation between tumor invasion and metastasis with some cysteine endopeptidases (particularly cathepsins B and L) has been observed [4]. Legumain is critical in the activation of cathepsin B, D, and H [2, 5, 6]. We and others showed legumain activates the zymogen progelatinase A, an important mediator of extracellular matrix degradation [1] [7]. The reported inhibitory effect of cystatins on tumor cells [8, 9] is consistent with the involvement of legumain and perhaps other cysteine proteases in tumor invasion and metastasis. Tumor invasion and metastasis are the major determinants of lethality, linked to 90% of human cancer deaths [10]. The high level of legumain expression by breast cancer cells and associated cells in the tumor microenvironment coupled with its unique specificity makes it an attractive candidate for prodrug therapy for breast cancer. We reported a doxorubicin derived prodrug prepared by incorporating a peptide extension of the amino group of doxorubicin resulting in an inactive compound unless hydrolyzed to leucine-doxorubicin by an asparaginyl endoprotease [1]. This compound, legubicin, resulted in complete tumor growth arrest and eradication in a model of human breast carcinoma without toxicity, such as weight loss, myelosuppression, and cardiac toxicity in contrast to doxorubicin treated mice. Doxorubicin and related compounds are the mainstay of breast cancer chemotherapy, however its application is limited by its toxicity. In contrast to doxorubicin, legubicin do not enter cells efficiently until activated by cell surface legumain in the tumor microenvironment. Pharmacokinetics analysis and tissue distribution study support tumor specific localization and activation of legubicin. As consequence, tumor accumulation and exposure to legubicin is greatly enhanced and organ exposure to chemotherapeutic agent is reduced. In organs containing cells that normally express legumain, such as kidney and liver, no injury was evident. Legumain expressed by kidney and liver cells are present in lysosomes and not

secreted. Extracellular legumain will be quickly inactivated by neutral environment in plasma and normal tissues. Legubicin demonstrated improved efficacy profile and therapeutic index vs doxorubicin by targeting neoplastic cells as well as endothelial cells and TAM in the tumor microenvironment therefore represents a promising candidate as a first line molecularly targeted chemotherapeutic agent replacing doxorubicn for the breast cancer treatment.

Body

The funding from W81XWH-05-1-0318 provided critical support for us to pursue research in translational medicine and experimental therapeutics in the area of breast cancer. We have developed a general strategy for targeted cancer therapy by creating cell-impermeable tumor microenvironment activated prodrugs (Liu 2003; Wu, Luo et al. 2006). The lead compound, LEG-3, is licensed by a commercial entity and is undergoing clinical development. To further validate legumain as a therapeutic target for caner, in collaboration with Dr. Rong Xiang, a vaccine strategy was successfully used to target tumor associated macrophages that express legumain (paper in appendix). A patent is filed to facilitate the possible development of inhibitors and prodrugs targeting legumain as therapeutic agents (abstract in appendix). The proposed research of this grant is accomplished and it also helped us to obtain significant preliminary data for future research. In its final year we will focus on the manuscripts describing inhibition of legumain in tumor microenvironment that led to suppression of tumor invasion/metastasis, and angiogenesis, as well as the function of legumain to protect cells from apoptosis.

1. Development of legumain activated prodrug, legubicin, as an anti-breast cancer treatment.

A. In vivo characterization of legubicins for preclinical efficacy, therapeutic protocol advancement and safety in a panel of rodent and human breast tumors in vivo. (Months 1-36)

An advanced candidate of the prodrug was tested in variety of breast cancer models and demonstrated significant effect against primary tumor growth and metastasis. The preclinical efficacy and safety was reported in a recent Cancer Research paper titled “Targeting Cell-impermeable Prodrug Activation to Tumor Microenvironment Eradicates Multiple Drug Resistant Neoplasms” will appear in Jan. 15th issue of Cancer Research. W81XWH-05-1-0318 provided critical support for the research and was gratefully acknowledged (PDF file of the paper is attached as appendix).

Most significantly, the prodrug demonstrated significant efficacy preventing metastasis in both spontaneous and experimental metastasis.

LEG-3 prodrug suppresses experimental and spontaneous metastasis.

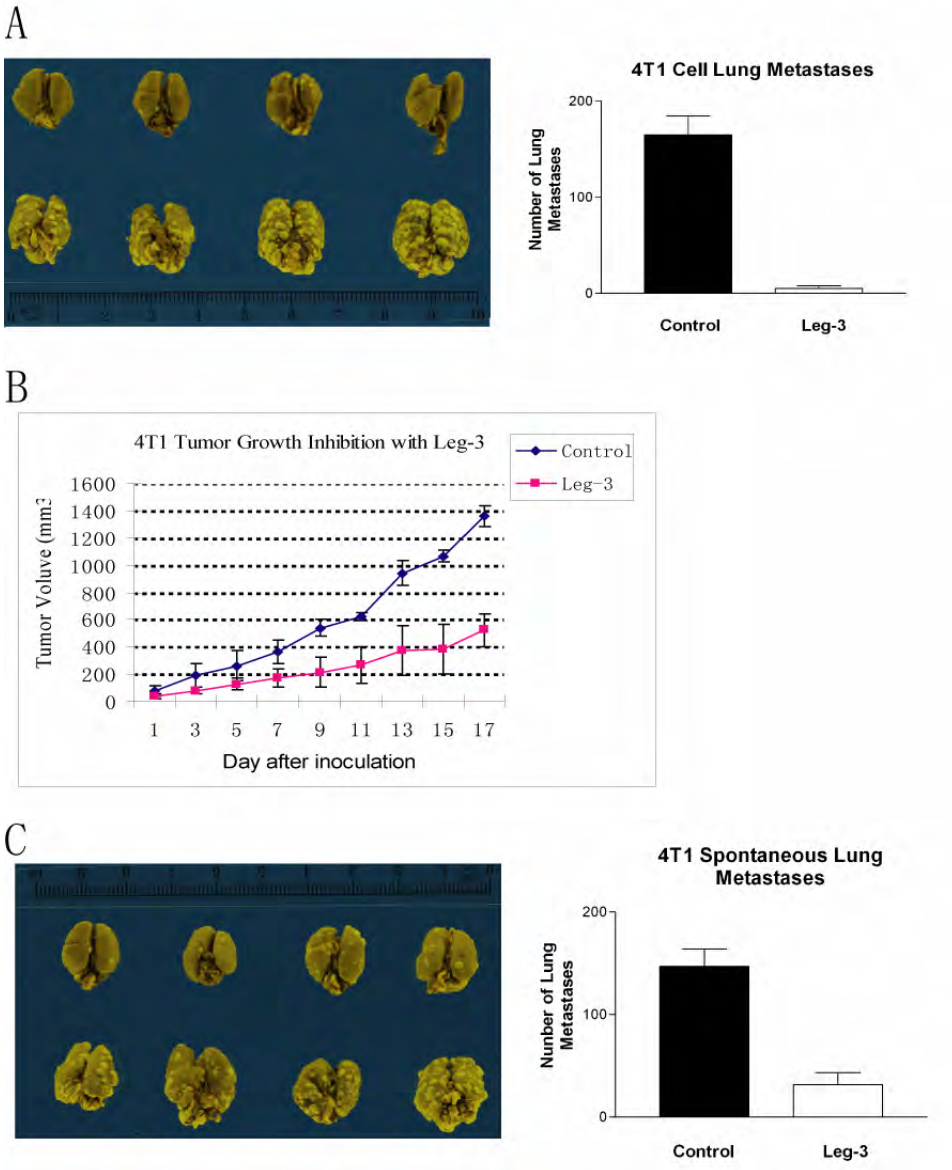


Figure 1. LEG-3 suppresses experimental and spontaneous metastasis. (A) Experimental lung metastasis in treatment group (top) versus control group (bottom). (p<0.005). (B) Mouse mammary carcinoma growth suppression by LEG-3 (n=8). (C) LEG-3 treatment significantly reduces spontaneous lung metastasis in 4T1 tumors. (p<0.005).

Treatment of LEG-3 prodrug significantly reduced experimental and spontaneous metastasis in 4T1 murine mammary carcinoma. The experimental metastasis models is generated by injecting 4T1 cells (1 x 10^5) suspended in 0.1 ml serum free medium into the tail vein of six-week-old female BALB/c mice. Treatment was given in 100-µl i.p. injections of either PBS alone (control group) or LEG-3 (100µg/100µl) daily for two weeks. The primary 4T1 models are generated by s.c. injection of 5 × 105 4T1 cells in the right flank of six-week-old BALB/c mice. Two different groups of four animals were treated between days 9 and 27 after tumor induction. Treatments were given in 100-µl i.v. injections of PBS alone (control group) or LEG-3 100µg/100µl in PBS by i.p. injection every two days for two weeks. Tumor volumes of treated animals were measured every two-day starting on day 9 by

microcaliper measurements (volume = width × length × width/2). As soon as the tumor volume reached 1400 mm^3 in the control groups (on day 30th), euthanasia was performed and lungs were removed and fixed in the Bouin's solution. Lung metastases were counted by anatomy microscope. Statistical significance between treatment groups was determined by two-tailed Student's t tests using Microsoft EXCEL software.

B. Legumain specificity explored through phage displayed substrate peptide library and synthetic peptide. (Months 1-6)

Due to the success with the current candidate compound and published work of other group, the construction of phage library will not be explored in this grant.

C. Improving chemistry and synthesis of legubicins, and efficient analysis of designed peptidyl and pseudopeptidyl derivative structures for improved efficacy and safety of legubicin analogues using in vitro assays. (Months 1-36)

We have synthesized different versions of the prodrug including dimer of the prodrug. And these candidate compounds have been tested for their in vitro and in vivo efficacies as proposed in the grant.

2. <u>Characterization of the molecular cell biology of legumain in breast cancer biology</u>.

A. Producing legumain knockdown and over-expression cells and investigate the effect of legumain knockdown and over-expression on breast cancer cells migration invasion in vitro. (Months 1-12)

We have produced multiple breast cancer cell lines that are over-expressing legumain as well as expressing legumain shRNA that suppresses legumain expression. Our data support the role of legumain in cell migration and invasion. Therefore this aim is accomplished. These work led to new hypothesis of mechanisms that legumain modulate tumor microenvironment.

B. Investigate the effect of legumain knockdown and over-expression on breast cancer invasion/metastasis in animal models. (Months 6-18)

Legumain expression is induced by hypoxia and occurs early in primary and metastatic tumors.

Hypoxia is an important factor in the tumor microenvironment. In both AMD-MB231 human breast cancer cells and MB21 human melanoma cells, legumain expression is induced in cells cultured under hypoxic conditions (1% O2), and its level continues to rise as the duration of hypoxic exposure increases (Figure 2A). HUVEC express very little legumain under normal culture conditions, but legumain expression is

significantly enhanced when the cells are cultured under hypoxic conditions for 72 hours (Figure 2A).

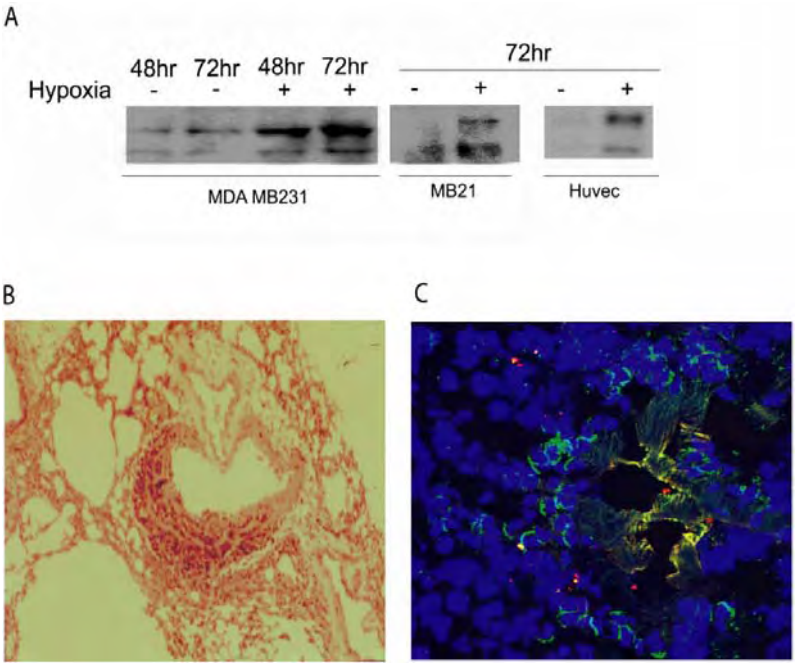


Figure 2. Legumain expression is induced by hypoxia and occurs early in metastatic sites. (A) Western blot analysis of legumain expression in cells under normoxic and hypoxic conditions. (B) H&E section of 4T1 breast carcinoma lung metastasis. (C) Immunohistochemical analysis of frozen section of 4T1 lung metastasis. Legumain is green and nuclei blue. The yellow color is auto-fluorescence of the wall of a bronchiole.

The 4T1 metastatic mouse mammary carcinoma metastasizes spontaneously to lung (Figure 2B). We examined legumain expression in lung metastatic sites. We found that legumain is expressed in metastatic sites when only a small number of tumor cells are present (Figure 2C). These data indicate up-regulation of legumain occur early during tumor development.

The αvβ3 integrins is cell surface receptor for legumain.

Legumain is distributed intracellularly as well as presented on cell surfaces in the tumor microenvironment. Legumain contains a RGD domain that is usually present in proteins that bind to integrins. To determine whether legumain binds to integrins, we performed immunoprecipitation with a panel of anti-integrin antibodies in MDA-MB231 cells that express low level of legumain in culture. Legumain is co-precipitated with anti-αv, αvβ3, β3, and β1 antibodies, suggesting αvβ3 is the candidate target (Figure 3A). To further characterize the interaction, co-immunoprecipitation was performed with anti-legumain antibody and detected with anti-αv integrin antibody (Figure 3B). In addition, legumain was detected when anti- αvβ3 was used as the immunoprecipitating antibody (Figure 3B).

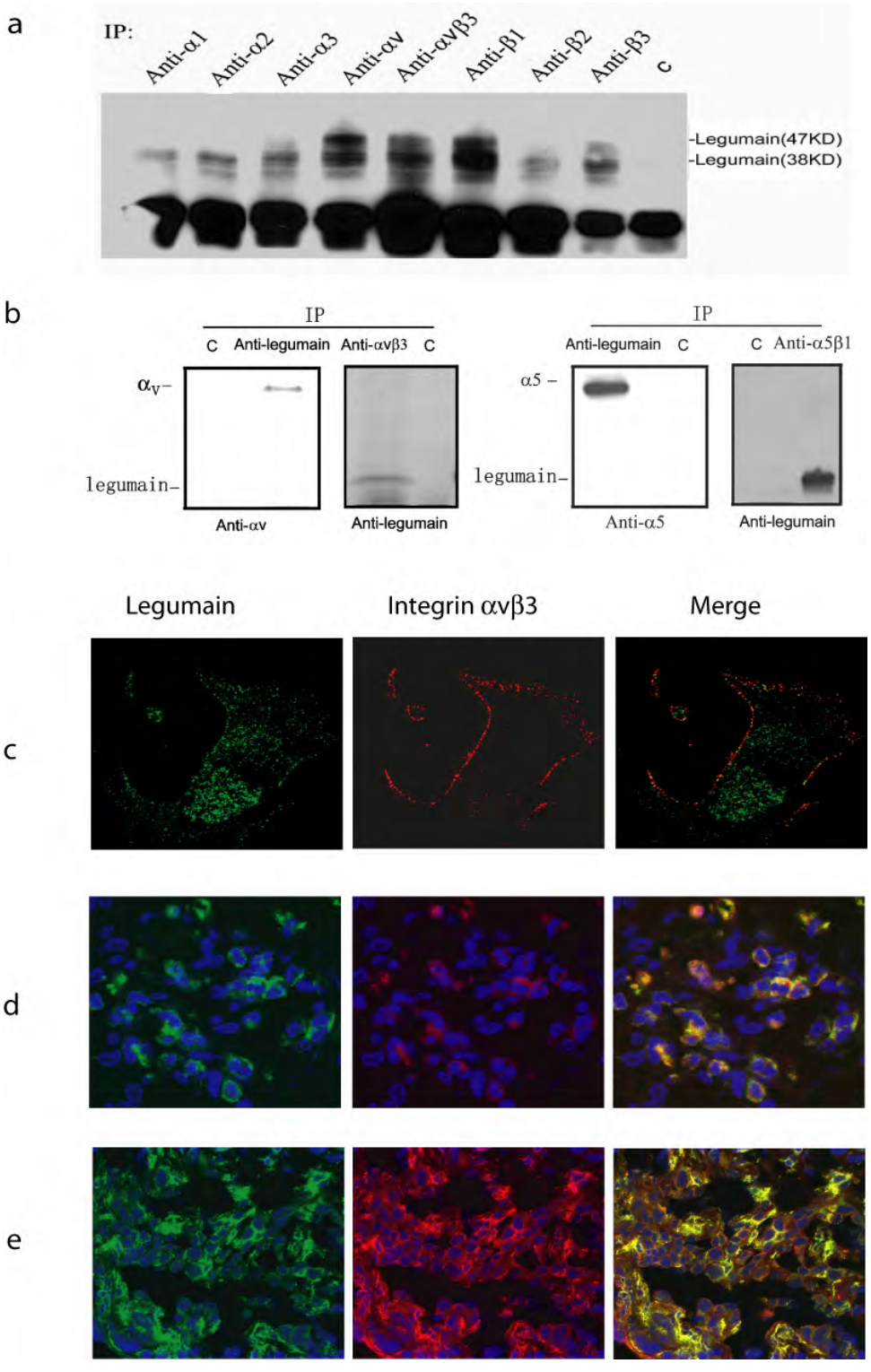


Figure 3. Legumain:αvβ3 form protease complex. (a) legumain is immuno-precipitated with a panel of anti-integrin antibodies. (b) Immuno-precipitation of legumain by anti αvβ3 antibody and αvβ3 by anti-legumain antibody. Same experiment with α5β1. (c) legumain:αvβ3 complex in MBA-MA231 cells. αvβ3 is red, legumain is green, nuclei is blue. And legumain:αvβ3 complex is yellow. Extensive legumain:αvβ3 colocalization in (d) Panc-1 human pancreatic carcinoma grow in nude mice and (e) MBA-MA-231 human breast cancer cells grown in nude mice. The αvβ3 is red, legumain is green, and cell nuclei are blue. The legumain:αvβ3 co-localization in complex is yellow.

Next, we performed Immunohistochemical staining of MDA-MB-231 human mammary carcinoma cells with both anti- αvβ3 and anti-legumain antibodies. Very little legumain and αvβ3 integrins are expressed in cell cultured under normal conditions. However, the level of legumain and αvβ3 integrins are both dramatically elevated under hypoxia and transported to cell surfaces where these proteins co-localize extensively. In migrating cells, the legumain:αvβ3 complex are predominantly present at the leading edge of the cells (Figure 3C). The presence of legumain:αvβ3 complex is also suggested by co-localization of legumain and αvβ3 integrins in Panc-1 and MBA-MD-231 tumors grown in nude mice (Figure 3d and e). Double staining of legumain and integrin β1 demonstrated extensive colocalization at the surface of lamellipodia (Fig 4) indicates legumain binds to additional integrins. Same staining patterns are observed for a panel of tumor and endothelial cells as well as macrophages.

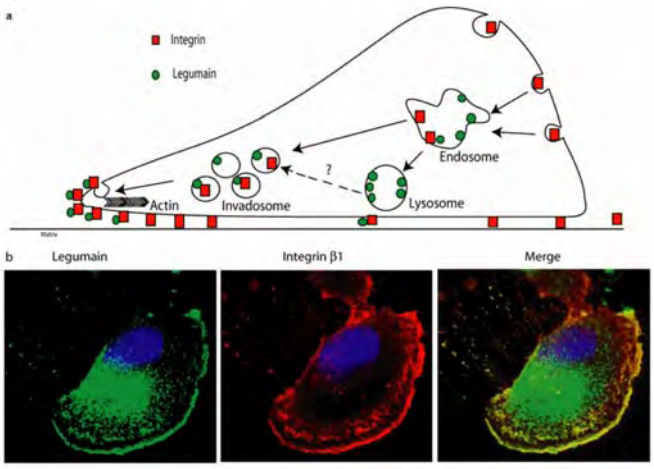
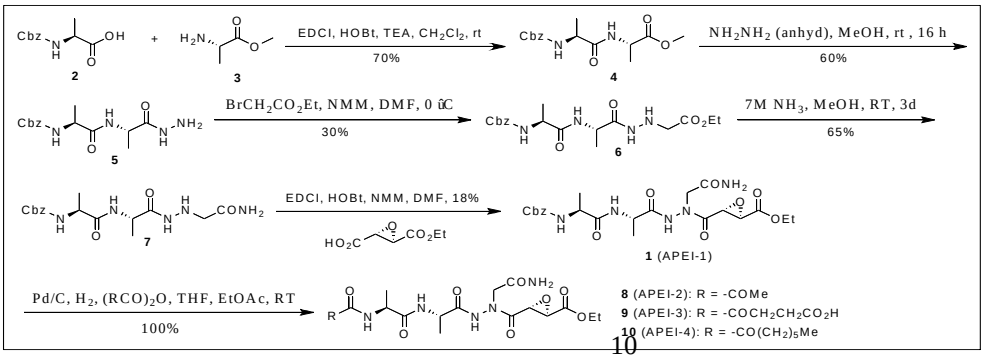


Figure 4. (a) A schematic presentation of legumain transportation to the front of invading cells.(b) Legumain (green) forms complex with integrins (red) on the surface of lamellipodia.

Asparaginyl endopeptidases inhibitors are highly specific and well tolerated.

Numerous azapeptides, composed of three or four amino acid residues, were prepared to mimic a naturally occurring epoxysuccinate derivative, E-64. These azapeptides are irreversible inhibitors of selected clan CD cysteine proteases that include caspases, legumain, gingipain, clostripain, and separase. Four asparaginyl endopeptidases inhibitors (AEPI) (Figure. 5a) were characterized using recombinant legumain in an amidolytic activity assay with fluorescent substrate (Figure 5b). Both AEPI-1 and AEPI-2 have IC50 at 20 nM and 21 nM respectively, the IC50 of AEPI-3 is 34 nM, while IC50 of AEPI-4 is 158 nM. The cytotoxicity of AEPI-1 was assessed in both wild type 293 cells (CC50, 320 μM) and 293 cells expressing legumain (CC50, 430 μM). No apparent cytotoxicity was observed until the 100 μM AEPI-1 was used (Figure 5c). AEPI-1 is highly specific to legumain and showed no activity towards other cysteine proteases (Figure 5d). Additional AEPI are being synthesized and analyzed.



Scheme 1. Synthesis of asparginyl endopeptidase

inhbitor (AEPI) 1.

Aza-peptide **1** was synthesized as described in the literature (Asgian, James et al. 2002)(Scheme 1). Briefly, dipeptide **4** was synthesized by coupling of Cbz-L-alanine and L-analine methyl ester, and then converted to the hydrazide **4** by treating with anhydrous hydrazine in methanol. The hydrazide **5** was alkylated with ethyl bromoacetate and the resultant product **6** underwent ammonolysis to afford amide **7**. The latter product was coupled with epoxy-succinic acid mono ethyl ester giving the desired compound **1**.

Compound **1** was designed to specifically inhibit legumain protease, which covalently reacts through the nucleophilic cysteine attack on the epoxide function in the former. Analogous to compound **1**, a modified compound, bearing an olefine instead of the epoxide function, has been designed, synthesized and evaluated. The latter compound undergoes Michael adduct formation with the legumain cysteine, and binds legumain with a comparable affinity as the compound **1** does. However, compound **1** is less toxic as compared to the olefin analog and therefore we are focusing on the epoxy molecule **1** and its cell-permeable derivative AEPI-5.

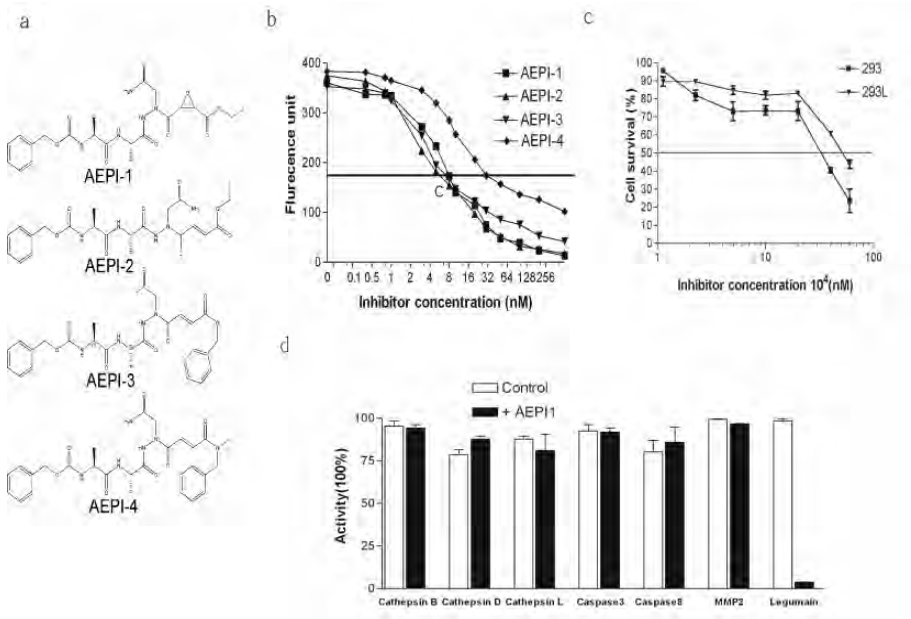


Figure 5. (a) Asparaginyl endopeptidases inhibitors (AEPI). (b) AEPI activity assessed by legumain amidolytic assay. (c) Cytotoxicity of AEPIs in wild type 293 cells and 293 cells expressing legumain. (d) AEPI specificity is assessed in activity assay of varied proteases as indicated. AEPI-1 is highly specific to legumain.

The αvβ3 integrins is a co-factor of cell surface legumain proteolytic activity.

The effect of binding to integrins on legumain enzymatic activity was assessed using a fluorescent substrate of legumain. With increasing concentration of αvβ3 integrin protein, there is a dramatic increase of legumain amidolytic activity suggesting αvβ3 integrins is not only a cell surface receptor of legumain it is also a co-factor of legumain activity (Figure 6a). Binding of legumain to αvβ3 increases legumain activity nearly 100 fold (Figure 6b). Further, binding of legumain to αvβ3 also affect its pH

dependency. The activity of legumain: αvβ3 complex are measured in different pH conditions and compared to that of legumain. Binding of legumain to αvβ3 shifts its peak activity from pH 5.2 to pH 6.0. The legumain: αvβ3 complex is active near pH 7, under which legumain alone will be inactivated (Figure 6b). These properties will significantly increase legumain activity at mild acidic conditions that exist in the extracellular space in the tumor microenvironment. The enhanced legumain amidolytic activity and shifted pH dependency indicate that the cell surface legumain:αvβ3 complex is likely the primary target enzyme of tumor microenvironment activated prodrug. We assessed the activity of legumain:αvβ3 against legumain physiologic substrate MMP2 (Figure 6c) and cathepsin L (Figure 6d). These results indicate that the legumain:αvβ3 complex is an important modulator of pericellular proteolysis during tumor invasive growth and angiogenesis (Figure 6e). AEPI treatment resulted in suppression of MMP-2 and cathepsin L in addition to legumain activity suggesting legumain activation of MMP-2 and cathepsin L is operational in a cellular context.

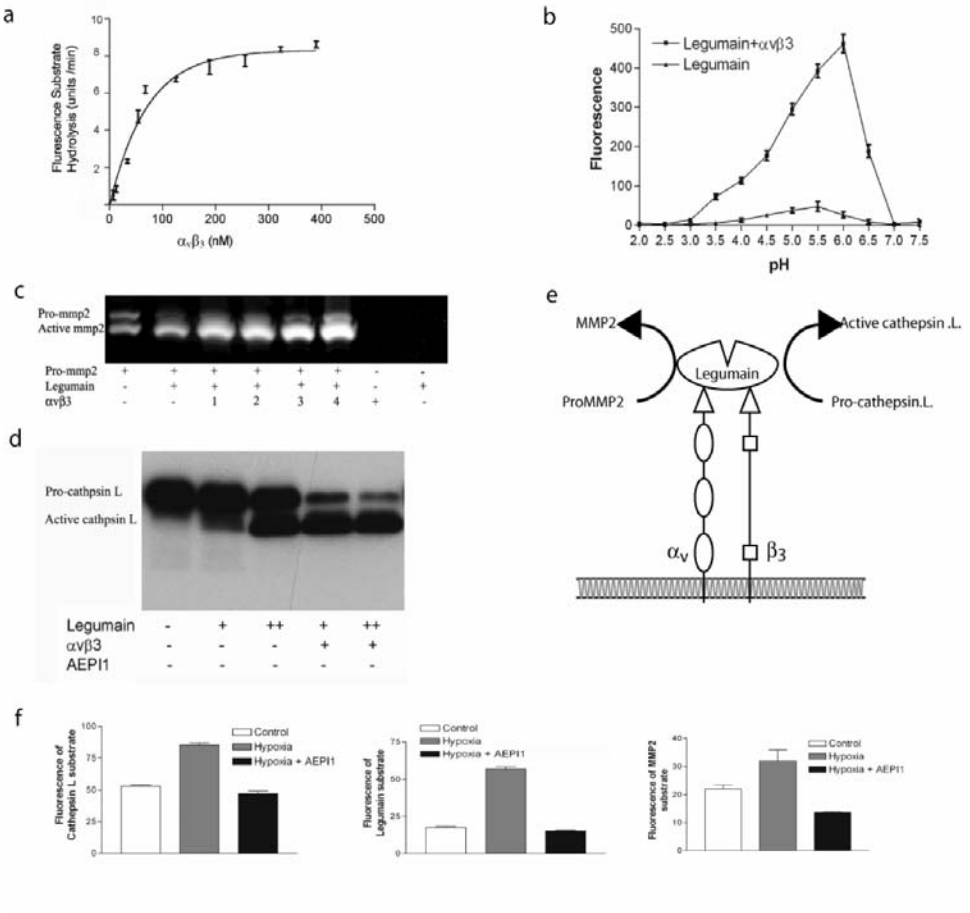


Figure 6. Integrin αvβ3 is co-factor of legumain activity. (a) Binding of αvβ3 increases the amidolytic activity of legumain in a dose dependent manner. (b) Comparison of asparaginyl endopeptidases activity of legumain and legumain: αvβ3 complex. The αvβ3 integrin also shift the pH dependency of legumain. (c) The αvβ3 increases its activity towards its physiologic substrate MMP2 as assessed using zymogram, (d)cathepsin L by Western blot. (e) Schematic representation of the legumain:αvβ3 complexe in intracellular trafficking and cell surface proteolysis. (f) Inhibition of legumain activity in tumor cells under hypoxia by AEPI-1 led to decreased activity of MMP-2 and cathepsin L

in addition to legumain activity as measured by their specific substrate respectively.

Asparaginyl endopeptidases activity of legumain is critical for angiogenesis.

The effect of AEPI-1 on endothelial cell function was assessed in vitro in Matrigel endothelial cell tube formation assay. AEPI-1 suppressed HUVEC tube formation in a dose dependent manner (Figure 7a). Inhibition of tube formation was apparent at 100 nM and was completely inhibited at 600 nM. In contrast, addition of recombinant legumain dramatically promoted and accelerated the tube formation (data not shown). HUVEC tube formation was observed as early as 5 hours after assay initiation versus 24 hours that are required for the vascular tubes to form under normal conditions. AEPI-1 also inhibited hypoxia induced tube formation and endothelial cell invasion in a invasion assay (Figure 7b). Next, the effect of AEPI-1 on angiogenesis was assessed in a mouse aortic sprouting assay. We tested the AEPI-1 in two settings. First, AEPI-1 was added at the start of the experiment to determine its effect on initiation of vessel sprouts. AEPI-1 inhibited FGF-2 induced vessel sprouts in a dose dependent manner (Figure 7c). In the second assay, AEPI-1 was added at the third day when sprouts are already formed. In this setting we try to determine whether asparaginyl endopeptidase activity is also required for the continued extension of vessel growth. Addition of AEPI-1 reduced extension of established vascular outgrowth suggesting the continued involvement of legumain in vessel development (data not shown). These observations are consistent with the ability of legumain to activate proteases and its association with integrins such as αvβ3 that are critical for angiogenesis.

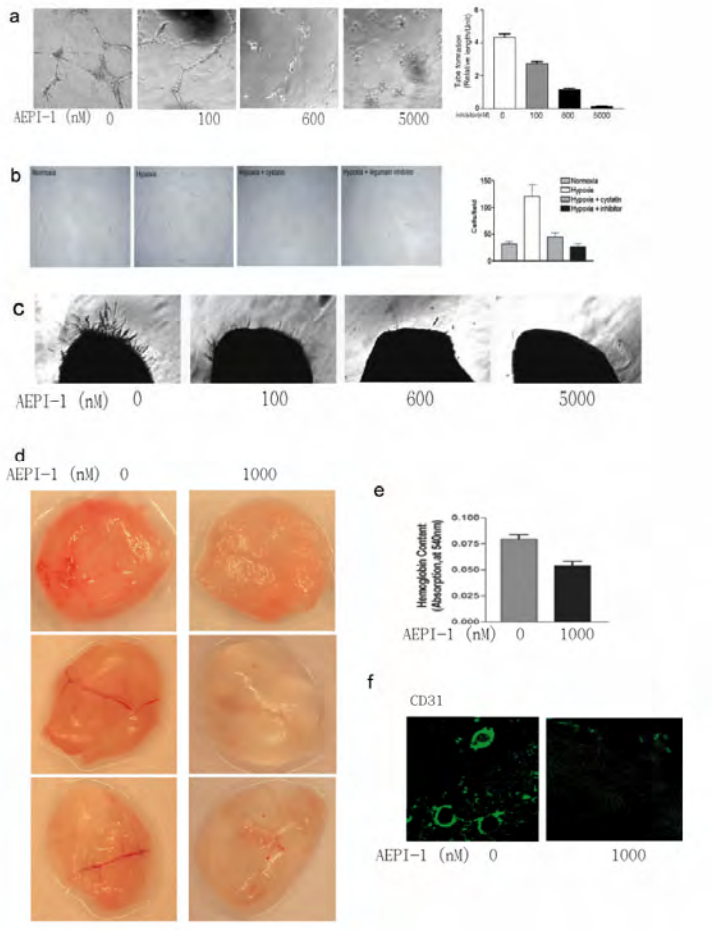


Figure 7. AEPI-1 inhibits angiogenesis in vitro and in vivo. (a) AEPI-1 inhibits HUVEC tube formation in vitro. (b) Hypoxia induced HUVEC tube formation and Invasion assays of under hypoxic conditions and their inhibition by cystatin and AEPI-1. (c) AEPI-1 inhibit mouse aorta ring sprouting assay ex vivo. (d) AEPI-1 inhibits angiogenesis in Matrigel plugs in vivo. (e) Vessel density is quantitated by

hemoglobin content in the plugs. (f) Representative CD31 positive vasculatures in Matrigel plugs.

We further investigated the effect of AEPI-1 on angiogenesis *in vivo* using a Matrigel plug assy. Matrigel contain FGF-2 was injected subcutaneously into Balb C nu/nu mice. AEPI-1 significantly inhibited FGF-2 induced vessel formation in the plug (data not shown) as indicated by gross appearance, hemoglobin content, and histology (Figure 7d). Next, we examined AEPI-1 effect on angiogenesis induced by cancer cells by including human breast cancer cells (MDA-MB-231) in the Matrigel. The cancer cells produced a spectrum of angiogenic factors and induced robust angiogenic vessel growth that is inhibited by AEPI-1 (Figure 7d, e, and f), indicating inhibition of asparaginyl endopeptidases activity affect angiogenesis induced by a wide range of angiogenic factors.

C. Legubicin action against tumor endothelial cells and tumor associated macrophages. (Months 18-36)

To target the cells in TME, metronomic dose of legumain-activated prodrug are use to treat 4T1 and CT26 tumors. Since legumain is expressed on the cell surfaces of both endothelial cells and TAM, both TAM and endothelial cells are targeted. TAM is the major producer of VEGR and FGF in tumors. The dosage of prodrug are determined from *in vitro* evaluation of endothelial cell and TAM susceptibility to prodrug and parent drug and adjusted accordingly to meet the local active drug concentration required to kill these cells. We follow an “antiangiogenic dosage” that, in principle, employs a low dose of chemotherapeutic agents that can be used more frequently and not allow the recovery of endothelial cells and angiogenesis. With a targeted prodrug, we administered every day and maintain a constant pressure on angiogenesis. To evaluate therapeutic efficacy, we will treat tumor bearing-mice (10/group) with prodrug or mock treatment (Figure 8A). The number of mice in each group is obtained based on the following calculation: for a SD of +/- 25% of the mean (equal variances), a *P* value equal to or less than 0.05.

Effect of abrogation of TAMs on the levels of angiogenic growth factors: TAMs can influence tumor metastasis in a variety of ways as they secrete a wide variety of tumor growth factors, pro-angiogenesis factors and tumor-associated enzymes that stimulate tumor angiogenesis, tumor growth and metastases. In an effort to assess whether the elimination of TAMs actually reduced the release of some of these factors, serum and tumor tissue cells will be collected from treated mice. Soluble factors are freshly extracted. An angiogenic protein arrays (Ray Biotech) will be used to assess the concentration of angiogenic factors (Figure 8. B and C).

A PCT patent titled “Inhibiting Tumor Cell Invasion, Metastasis, and Angiogenesis” was filed Nov. 29, 2006 (PDF file is attached).

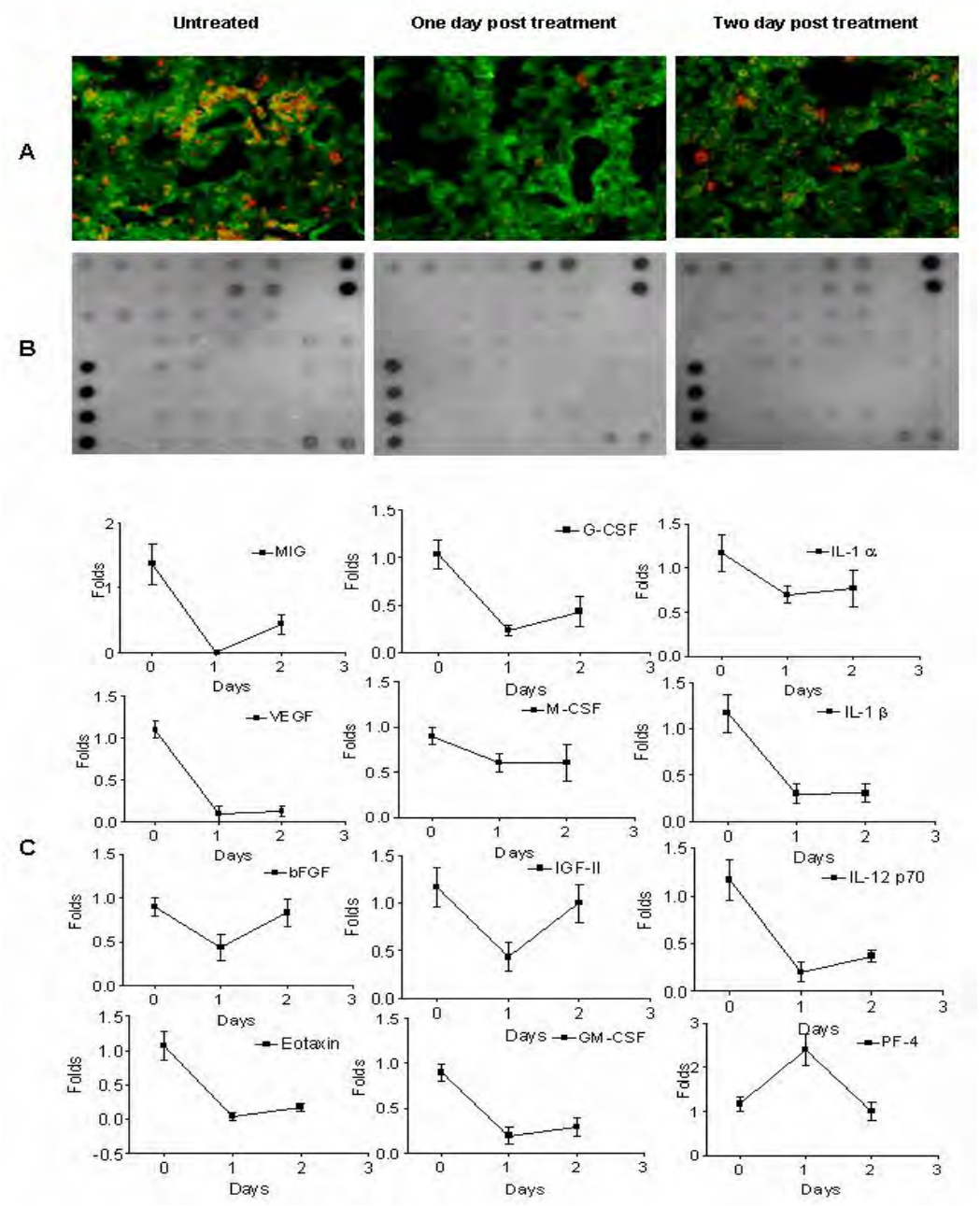


Figure 8. LEG-3 treatment kills TAM and reduces the level of angiogenic factors in tumors. A. Immunohistochemical staining of CT26 tumors treated with LEG-3. Legumain positivity is green and TAMs are identified by CD68 staining that is red. B. Example of angiogenic factor arrays. C. Level of angiogenic factors during LEG-3 treatment.

Key Research Accomplishments

1. Identification of legumain:integrin complex and its function in breast cancer progression and metastasis.

2. Demonstrated efficacy of TME activated prodrug, legubicin, against drug resistant breast cancers.

3. Elucidation of the mechanism of legumain:integrin complex in promoting metastasis/invasion and angiogenesis as well as serve as enzymatic target for legubicin.

4. Demonstrate inhibition of legumain:integrin complex activity in the TME suppress tumor invasive growth, angiogenesis, and metastasis.

Reportable Outcomes

1. Prove of principle of tumor microenvironment activated prodrugs and in vivo demonstration of prodrug efficacy against drug resistant breast cancers (paper published in Cancer Research and JCI).

2. The prodrug demonstrated significant anti-cancer efficacy and the data generated from the first year formed a base of a new provisional patent.

3. Funding of DOD breast cancer synergistic grant in 2007 based on preliminary data and collaboration supported by this award.

4. Funding of a NIH R01 partly based on preliminary data generated by this award.

5. License of IP to Affinity Pharmaceuticals Inc. to further develop legubicin as a breast cancer therapy.

Conclusions

The local tumor microenvironment differs greatly from that of other tissues. One key character is that it is enriched in proteolytic activity. Cell surface proteases, such as legumain:integrin complex, play important role in cancer progression such as invasion/metastasis and angiogenesis. The over-expression of these cell surface proteases are ideal physical as well as functional targets to activate prodrugs in the tumor microenvironment as demonstrated by data generated with the support of this grant.

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Appendices

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INHIBITING TUMOR CELL INVASION, METASTASIS AND ANGIOGENESIS

This application claims priority to the filing date of U.S. Provisional Application Ser. No. 60/740,575, filed November 29, 2006, which is incorporated herein by reference in its entirety.

This application is related to PCT Application Ser. No. PCT/US2004/017157 filed May 28, 2004, which claims benefit of U.S. Application Ser. No. 60/474,840 filed May 29, 2003, both of which are incorporated by reference herein in their entireties.

Statement of Government Rights

The invention was made with the support of a grant from the Government of the United States of America (CDMRP Grant Numbers W81XWH-05-1-0091 and W81XWH-05-1-0318 from the Department of Defense). The Government may have certain rights to the invention.

Field of the Invention

The present invention relates to methods for treating and/or inhibiting tumor cell invasion, metastasis and/or angiogenesis as well as increasing apoptosis in cancer cells by administering asparaginyl endopeptidase inhibitors. In some embodiments, the invention relates to inhibitors of proteases that are expressed under the hypoxic conditions of the tumor microenvironment. In other embodiments, the invention relates to prodrug agents that become activated by the proteases that are expressed in the tumor microenviroment. The prodrugs become active within the tumor microenvironments of primary and metastatic tumor sites, for example, at the surface of, cancerous cells and tumor stomal cells that express proteases.

Background of the Invention

The following description includes information that may be useful in understanding the present invention. It is not an admission that any of the information

provided herein is prior art, or relevant, to the presently described inventions, or that any publication or document that is specifically or implicitly referenced is prior art.

According to the National Cancer Institute, since 1990 over 17 million people have been diagnosed with cancer, and an additional 1,334,100 new cancer cases are expected to be diagnosed in 2003. About 556,500 Americans are expected to die of cancer in 2003, more than 1500 people every day. Cancer is therefore the second leading cause of death in the United States, exceeded only by heart disease. The National Institutes of Health estimate the overall costs of cancer in the year 2002 at $171.6 billion (Cancer Facts & Figures, 2003). Clearly, cancer is an enormous problem, and more effective cancer treatments are needed.

Two characteristic features of malignant cells are the ability to invade normal tissues and the ability to spread to distant sites. Tumor metastasis and invasion are the main cause of cancer mortality. Malignant cells can spread by several routes including direct local invasion, by the lymphatics or by capillaries. Local invasion is accomplished by an increase of tumor cell mobility and by production of proteases that destroy the normal extracellular matrix and basement membranes. Once the tumor cells escape from their normal boundaries, they are free to enter the circulation through the capillaries and the lymphatic system. The need for methods to prevent tumor invasion and metastasis is critical and constitutes a major goal in the effort to develop effective therapeutic interventions against cancer.

In addition, many cancer cells are capable of inducing angiogenesis. To form blood vessels, angiogenic endothelial cells share some of the same biochemical mechanisms that are used by cancer cells to invade local tissues.

Current cancer treatments generally involve the use of surgery, radiation therapy, and/or chemotherapy. However, these treatments all involve serious side effects. For example, surgery can be complicated by bleeding, damage to internal organs, adverse reactions to anesthesia or other medicines, pain, infection, and slow recovery. Radiation therapy can damage normal cells and can cause fatigue. For many people, chemotherapy is the best option for controlling their cancer. However, chemotherapy can also damage normal cells such as bone marrow and blood cells, cells of the hair

follicles, and cells of the reproductive and digestive tracts. Chemotherapy can also cause nausea, vomiting, constipation, diarrhea, fatigue, changes to the nervous system, cognitive changes, lung damage, reproductive and sexual problems, liver, kidney, and urinary system damage, and, especially with the use of the chemotherapeutic agent doxorubicin, heart damage. Long-term side effects of chemotherapy can include permanent organ damage, delayed development in children, nerve damage, and blood in the urine. Thus, the use of the chemotherapy for cancer treatment is not without serious side effects.

Most agents currently administered to a patient are not targeted to the site where they are needed, resulting in systemic delivery of the agent to cells and tissues of the body where the agent is unnecessary, and often undesirable. Such systemic delivery may result in adverse side effects, and often limits the dose of an agent (*e.g.*, cytotoxic agents and other anti-cancer agents) that can be administered. Accordingly, a need exists for agents and methods that specifically target cancerous cells and tissues.

Thus, it would be desirable to be able to direct various agents to cancer cells and to the tumor microenvironment so as to be able to decrease the dosage of the agents given and to decrease the systemic toxicity and side effects associated with these agents.

Summary of the Invention

According to the invention, the tumor microenvironment creates conditions that induce expression of certain genes, including proteases that are active almost exclusively in the tumor microenvironment. For example, an unexpectedly high level expression of asparaginyl endopeptidases, including legumain, is present in a wide variety of cancer cells, particularly those involved in metastasis. Other proteases that are active in the tumor microenvironment include prostate specific membrane antigen (PSMA)(a carboxypeptidase), fibroblast activation protein (FAP) (a serine peptidase), cathepsin B (a cysteine protease), cathepsin X (a cysteine protease), urokinase-type plasminogen activator (uPA)(a serine protease), tissue factor VIIa (TF VIIa)(a serine protease), matriptase (a membrane-bound serine protease) and Factor XIII. As

described herein, tumor-specific protease expression occurs early in the development of cancer cell invasion, just as metastasis begins, and under the hypoxic conditions associated with invasive tumor growth. Moreover, legumain is directly involved in and actually inhibits the cascade of activities that leads to cellular apoptosis, particularly in cancer cells where legumain is highly expressed.

The tumor specific proteases described herein are cell surface associated proteases. These proteases function in protease networks that play critical roles in modulating extracellular matrix proteins. For instance, certain cancers may employ more cysteine proteases than matalloproteinas or serine protease and verse versa. However the matrix modifying function of each of these proteases is indispensable for tumor metastasis and invasive growth.

According to the invention, the combined use of protease activity imaging agents, protease inhibitors, and/or prodrugs described herein represents an integrated precision-guided cancer therapeutic system. The combined use of protease inhibitor and prodrugs are also envisioned, although in some embodiments the prodrug and protease inhibitor targeting same protease are used at different times during the therapeutic regimen.

Also as shown herein, legumain activity is substantially increased on the surface of tumor cells by cell-surface association with integrins, indicating that integrins are co-factors for legumain. Moreover, legumain can activate metalloproteinases (e.g. MMP-2) and cathepsins (e.g., cathepsins B, H and L), which are all proteases involved in promoting tumor cell invasion and metastasis. In addition, asparaginyl endopeptidase expression is also associated with reduced cancer cell apoptosis and increased angiogenesis. Therefore, expression of, and activity by, certain proteases, including legumain, PSMA, FAP, cathepsin B, cathepsin X, uPA, tissue factor VIIa, matriptase (a membrane-bound serine protease) and Factor XIII are cancer and angiogenesis markers and constitute indicia of tumor cell metastasis. The invention therefore provides agents to treat undesirable angiogenesis, tumor cell invasion, tumor cell metastasis and other such cancerous conditions, particularly those conditions involving cells and tissues that express these proteases.

Many tumor cells are largely resistant to chemotherapy, for example, because the chemotherapeutic agents employed are only active against a subset of the tumor cells that comprise a cancerous condition. According to the invention, stromal cells in the tumor microenvironment, such as endothelial cells or tumor associated macrophages (TAMs), can be targeted by the agents of the invention to effectively treat these drug-resistant tumor cell types. This strategy is also effective for reducing the expression and/or activity of molecules in the tumor microenvironment that attract TAMs and other tumor-associated cells that facilitate tumor growth and invasion. TAMs consist of a polarized M2 (CD206+, F4/80+) macrophage population. TAMs also possess poor antigen presenting capacity and effectively suppress T cell activation. In fact, TAMs actually promote tumor cell proliferation and metastases by producing a wide range of growth factors, pro-angiogenesis factors, metalloproteinases and the like. TAMs also partake in circuits that regulate the function of fibroblasts in the tumor stroma and are particularly abundantly expressed in the tumor stroma.

According to the invention, TAMs express high levels of certain proteases, including legumain, in the tumor microenvironment. In contrast, classical macrophages of the M1 phenotype, that perform key immune-surveillance functions, do not express legumain. Consequently, targeted elimination of TAMs does not interfere with the biological functions of normal (MI) macrophages, including cytotoxicity and antigen presentation. Thus, one aspect of the invention involves targeting legumain-expressing TAMs with prodrugs and/or proteases inhibitors to destroy TAMs and/or inhibit their function.

TAM and endothelial cells are non-transformed cells therefore will not develop drug resistance that is common among malignant cancers. Thus, low dosages of the prodrugs and/or protease inhibitors can be employed when targeting these TAM and endothelial cells. This will down regulate a wide variety of tumor growth factors, pro-angiogenesis factors and enzymes released by these macrophages and lead to inhibition of tumor angiogenesis as well as invasive growth and metastasis.

One aspect of the invention is a method of treating cancer in a mammal by administering to the mammal an effective amount of a prodrug or an inhibitor of a

protease that is expressed in the tumor microenvironment. According to the invention, treatment of cancer can involve killing tumor cells, reducing the growth of tumor cells and reducing the growth or function of tumor stromal cells in a mammal. Examples of stromal cells that can be treated by the methods of the invention include tumor-associated macrophages and endothelial cells. Treatment of of cancer can also involve promoting apoptosis of cells that express legumain. As shown herein, expression of legumain inhibits apoptosis and cancer cells that exhibit high levels of legumain expression resist apoptosis. Treatment of cancer can also involve inhibiting angiogenesis of a tumor in a mammal.

Another aspect of the invention is a protease-activated prodrug that is tumoricidal *in vivo*, wherein the protease is a protease that is expressed in the tumor microenvironment (e.g., under the hypoxic conditions of tumor microenvironments). These protease-activated prodrugs have reduced side effects and toxicity relative to currently available chemotherapeutics. While the present prodrugs are useful for treating cancer, they are also useful to treat other conditions and cellular environments that express proteases. For example, certain non-transformed cells support tumor growth and invasion and, as described herein also express proteases when present in the tumor microenvironment. Hence, the present prodrugs can be used to target and kill not only cancer cells but also the cells that support tumor growth and tumor cell metastasis.

A prodrug compound of the invention includes a drug molecule linked to a peptide, wherein the peptide has an amino acid sequence that is specifically recognized by a protease expressed in a tumor microenvironment. Thus, the peptide includes at least two linked amino acids, wherein at least one of the two linked amino acids is an amino acid that is specifically recognized by a tumor-specific protease and forms part of a cleavage site for the protease. For example, legumain is a protease that is specifically expressed in tumor cells and cells that support tumor growth and metastasis (e.g. tumor associated macrophages). Legumain is an asparaginyl protease that specifically recognizes asparagine-containing peptides and cleaves peptides that contain asparagine (Asn). Some of the prodrugs of the invention are therefore designed

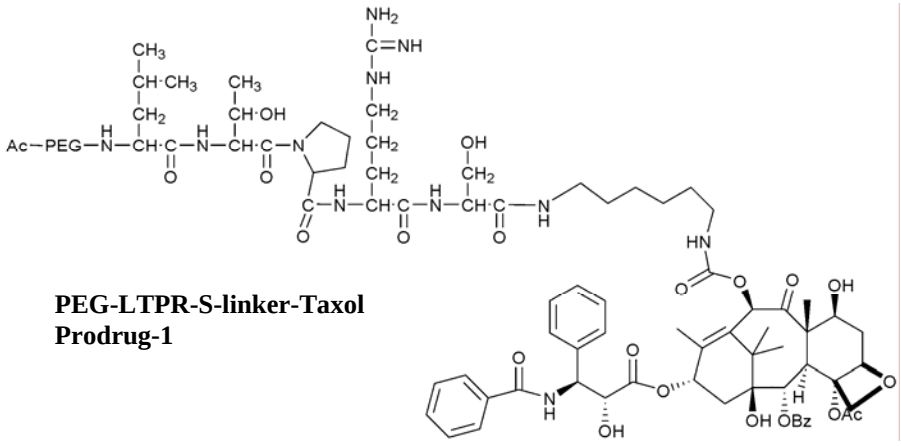
to be activated by legumain. Legumain cleaves the peptide of the present prodrugs at the site of the Asn to generate an active drug from the prodrug. Prior to cleavage, the prodrug is substantially non-toxic to normal animal cells, whereas after cleavage, the drug is an active drug that can have a beneficial effect upon an animal to which it is administered.

Prodrugs of the invention have the general structure:

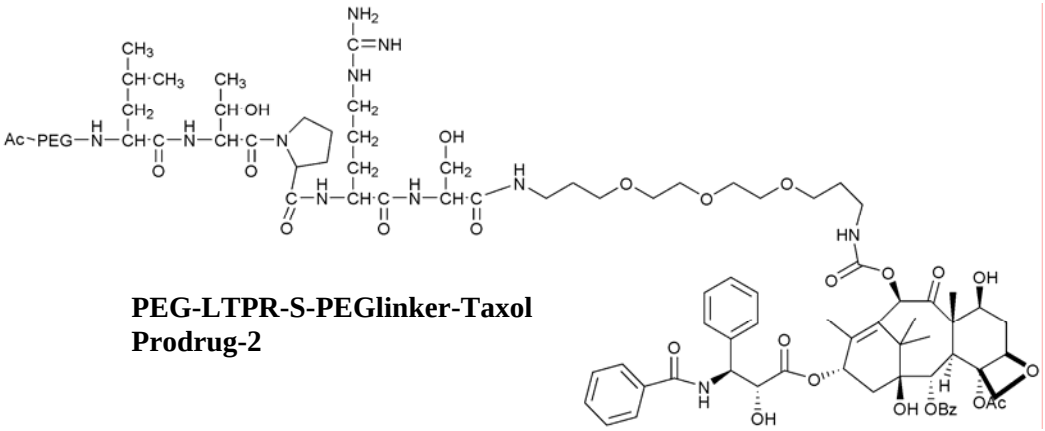
R_1-peptide-drug

wherein R_1 is hydrogen, a hydrophilic group, a hydrophobic group, a photosensitizing agent, a label or an amino protecting group; and drug is any therapeutic agent. In some embodiments, the R_1 groups of the present prodrugs are not hydrophobic groups or labels. The R_1 and drug moieties can be linked directly to the peptide or they can be linked to the peptide through a linker or spacer molecule. Such a linker of spacer can be an alkylene, a sugar or an oligosaccharide.

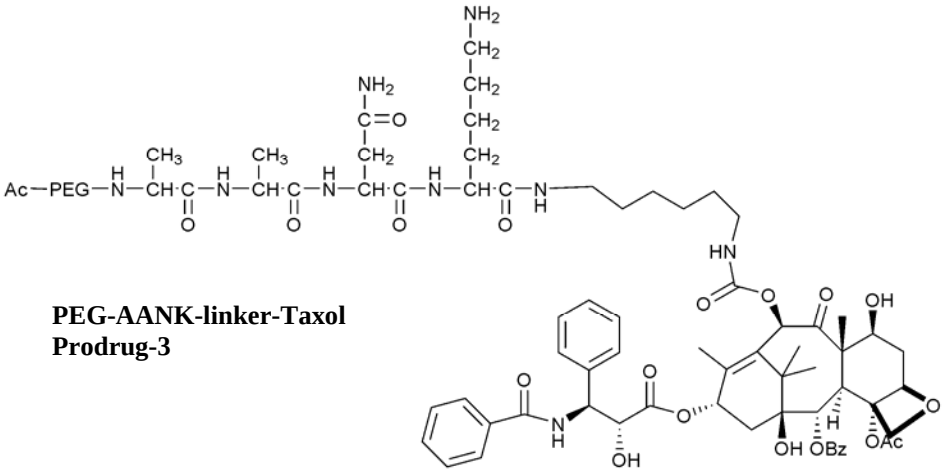
Specific examples of prodrug compounds of the invention include, for example, taxol, paclitaxel, doxorubicin containing prodrugs,including those shown below:



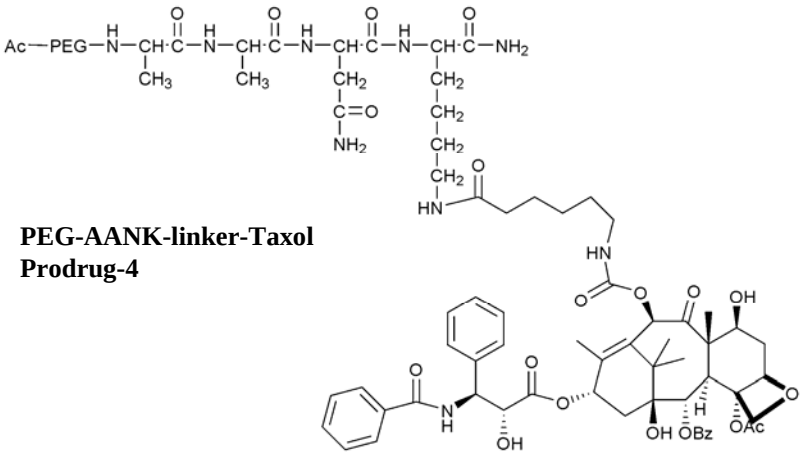
PEG-LTPR-S-linker-Taxol Prodrug-1



PEG-LTPR-S-PEGlinker-Taxol
Prodrug-2

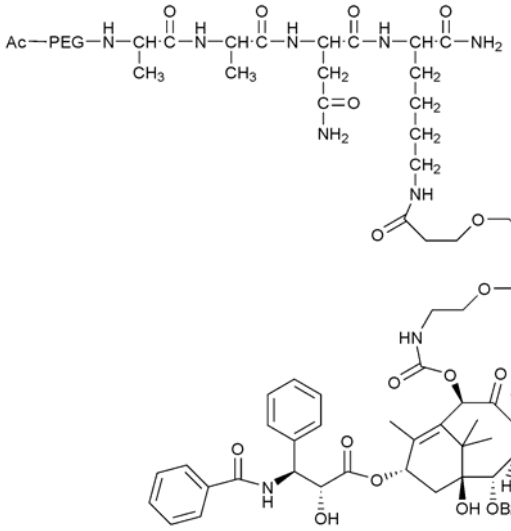


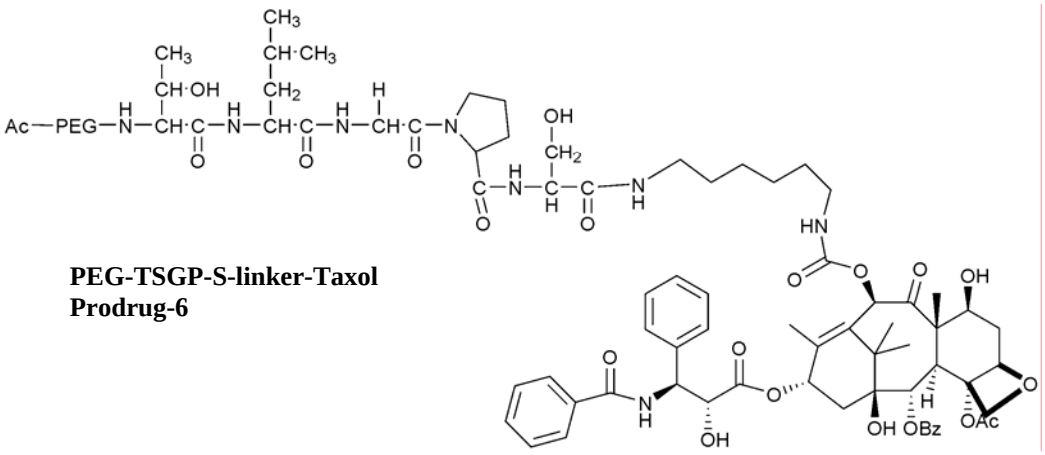
PEG-AANK-linker-Taxol
Prodrug-3



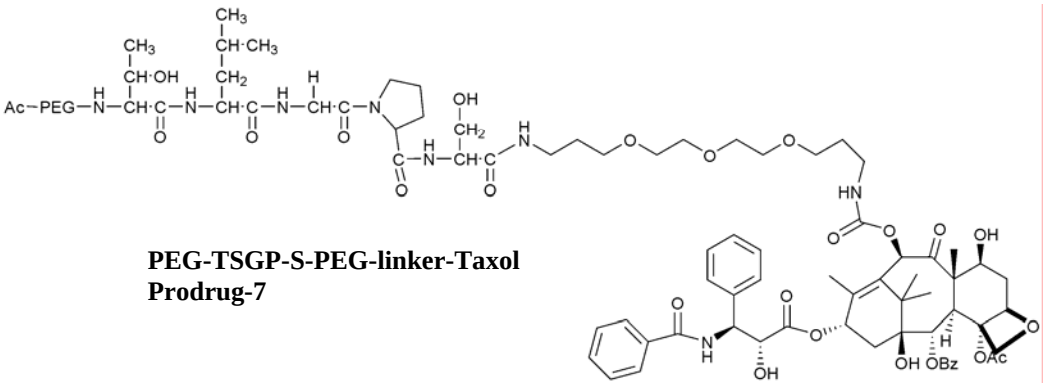
PEG-AANK-linker-Taxol
Prodrug-4

PEG-AANK-PEGlinker-Taxol (Prodrug-5):





PEG-TSGP-S-linker-Taxol
Prodrug-6



PEG-TSGP-S-PEG-linker-Taxol
Prodrug-7

Prodrug-1 and prodrug-2 are activated by serine protease TF VIIa. Prodrug-3, prodrug-4 and prodrug-5 are activated by legumain. Additional legumain prodrugs include LEG-2 (*N*-Succinyl- -alanyl-L-threoinyl-L-Asparaginyl-L-Leucyl-Doxorubicin)

and LEG-3 (*N*-Succinyl- -alanyl-L-alanyl-L-Asparaginyl-L-Leucyl-Doxorubicin), whose structures are shown in FIG. 9A. In some instances, the prodrugs of the invention do not include LEG-2 or LEG-3, because the inventor has filed a separate application on these compounds. Prodrug-6 and prodrug-7 are activated by fibroblast activation protein (FAP).

The drug employed is any drug whose action is diminished or blocked by attachment of a peptide to the drug. The ability of the drug to enter cells is diminished, inhibited or blocked by attachment of the peptide and hydrophilic groups. Such hydrophilic groups are generally included to facilitate water-solubility and cell impermeability. Hydrophilic groups are generally attached to the peptide so that the function of the drug is not inhibited or blocked by the hydrophilic group once the peptide is cleaved from the prodrug to yield the drug.

In some embodiments, the drug can be a cytotoxin or a photosensitizing agent. Such a cytotoxin can be aldesleukin, 5-aminolevulinic acid, bleomycin sulfate, camptothecin, carboplatin, carmustine, cisplatin, cladribine, lyophilized cyclophosphamide, non-lyophilized cyclophosphamide, cytarabine, dacarbazine, dactinomycin, daunorubicin, diethyistilbestrol, epoetin alfa, esperamycin, etidronate, etoposide, filgrastim, floxuridine, fludarabine phosphate, fluorouracil, goserelin, granisetron hydrochloride, idarubicin, ifosfamide, immune globulin, interferon alpha-2a, interferon alpha-2b, leucovorin calcium, leuprolide, levamisole, mechiorethamine, medroxyprogesterone, melphalan, methotrexate, mitomycin, mitoxantrone, octreotide, ondansetron hydrochloride, paclitaxel, pamidronate, pegaspargase, plicamycin, protoporphyrin IX, sargramostim, streptozocin, taxol, thiotepa, teniposide, vinblastine, or vincristine. In some embodiments, the drug is doxorubicin, 5-aminolevulinic acid, protoporphyrin IX, taxol or paclitaxel.

In one embodiment, the prodrug is activated by asparaginyl proteases (e.g., legumain) and has a peptide amino acid sequence comprising SEQ ID NO:3:

$$R_1\text{-}(Xaa1)_n\text{-}Xaa2\text{-}Asn\text{-}(Xaa3)\text{-drug}$$

wherein:

R_1 is hydrogen, a hydrophilic group, a hydrophobic group, a photosensitizing agent, a label or an amino protecting group;

n is an integer of about 0 to about 50;

Xaa1 and Xaa2 are separately any amino acid;

Xaa3 is either nothing or an amino acid that has no substantial effect on the activity of the drug; and

the drug employed is a drug whose action is diminished or blocked by attachment of a peptide to the drug.

In some embodiments, the R_1 groups of the present prodrugs are not hydrophobic groups or labels. For example, when cytotoxic drugs are part of the prodrug, a hydrophilic group is preferably used for R_1 to limit cell uptake by non-target cells.

Examples of peptide sequences that may be used in the prodrugs of the invention include amino acid sequence Asn-Leu, Ala-Asn-Leu, Thr-Asn-Leu, Ala-Ala-Asn-Leu (SEQ ID NO:5), Ala-Thr-Asn-Leu (SEQ ID NO:6), and Boc-Ala-Ala-Asn-Leu (SEQ ID NO:4). Examples of prodrugs provided by the invention include Boc-Ala-Ala-Asn-Leu-doxorubicin (SEQ ID NO:7), succinyl-Ala-Ala-Asn-Leu-doxorubicin (SEQ ID NO:8), N-(-*t*-Butoxycarbonyl-Ala-Thr-Asn-Leu)doxorubicin (SEQ ID NO:9), N-(Succinyl-Ala-Thr-Asn-Leu)doxorubicin (SEQ ID NO:10), N-(-*t*-Butoxycarbonyl-Ala-Asn-Leu)doxorubicin (SEQ ID NO:11), N-(Succinyl-Ala-Asn-Leu)doxorubicin (SEQ ID NO:12), N-(-*t*-Butoxycarbonyl-Thr-Leu)doxorubicin (SEQ ID NO:13), N-(Succinyl-Thr-Leu)doxorubicin (SEQ ID NO:14),

As described herein a hydrophilic R_1 group (sometimes abbreviated herein as “Hyd”) facilitates prodrug and inhibitor water solubility and inhibits cell uptake and tissue retention of the prodrug before activation and of the inhibitor before binding to a protease (e.g. the legumain:integrin complex). A variety of hydrophilic protecting groups can be utilized. Hydrophilic R_1 groups of the invention can be sugars (monosaccharides and disaccharides), dicarboxylic acids (e.g., succinate, malate, fumarate, oxaloacetate, citrate, isocitrate), glycans, polyalkylene oxides, lower alkyl carboxylates, carboxyalkyls, carboxyalkylene carboxylates, charged amino acids (e.g., any of the hydrophilic, acidic, basic and polar amino acids described herein) and the

like. In some embodiments the protecting group is a hydrophilic amino protecting group. Specific examples of R_1 groups that can be used include glucuronide, succinyl, polyethylene glycol (PEG) or glutathione. Hydrophobic groups can used if cellular uptake of the drug is desired. Hydrophobic groups that can be used include those listed herein.

The invention also provides a protease inhibitor having including formulae **III**, **IV**, **V** or **VI**:

R_1-$(Xaa4)_n$-Asn-Y **III**

R_1-$(Xaa4)_n$-Xaa5-Y **IV**

R_1-Xaa4-azaAsn-Y **V**

R_1-Xaa4-azaXaa5-Y **VI**

wherein:

wherein R_1 is hydrogen, a hydrophilic group, a hydrophobic group, a photosensitizing agent, a label or an amino protecting group;

n is an integer of about 2 to about 5;

each Xaa4 and Xaa5 is an amino acid or an amino acid mimetic;

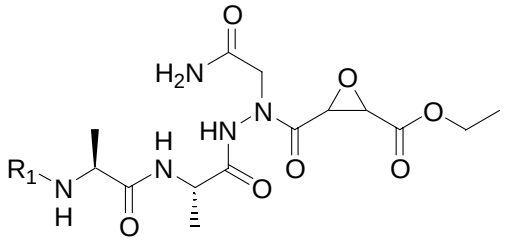
Y is alkyl, alkenyl epoxide, fluoromethylketone or a Michael acceptor, optionally substituted with 1-3 halo or hydroxy, alkylamino, dialkylamino, alkyldialkylamino, or cycloalkyl, alkylcycloalkyl, alkenylcycloalkyl, aryl; (C_5 -C_{12})arylalkyl or (C_5 -C_{12})arylalkenyl,

wherein the aryl groups of the arylalky or arylalkenyl can be 0-4 heteroatoms selected from N, O and S, and are optionally substituted with halo, cyano, nitro, haloalkyl, amino, aminoalkyl, dialkylamino, alkyl, alkenyl, alkynyl, alkoxy, haloalkoxy, carboxyl, carboalkoxy, alkylcarboxamide, (C_5 -C_6)aryl, --O-(C_5 -C_6)aryl, arylcarboxamide, alkylthio or haloalkylthio; and

wherein each of the inhibitors of formulae **III**, **IV**, **V** and **VI** bind to a protease expressed in a tumor microenvironment.

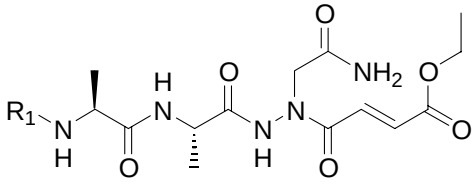
Examples of asparaginyl endopeptidase inhibitors (AEPIs) that may be used in the methods of the invention include the following:

AEPI-1 is R_1-Ala-Ala-AzaAsn-(S,S)-EPCOOEt, a compound of the structure:



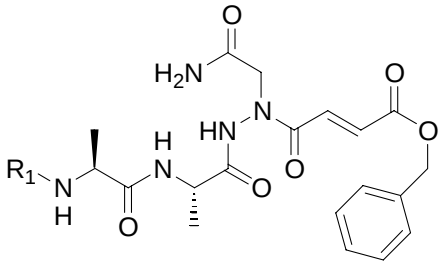
AEPI-1

AEPI-2 is R_1-Ala-Ala-AzaAsn-CH=CH-COOEt, for example, a compound of the structure:



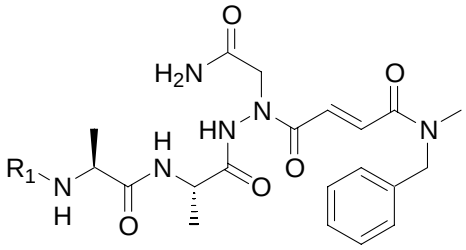
AEPI-2

AEPI-3 is R_1-Ala-Ala-AzaAsn-CH=CH-COOBzl, for example, a compound of the structure:



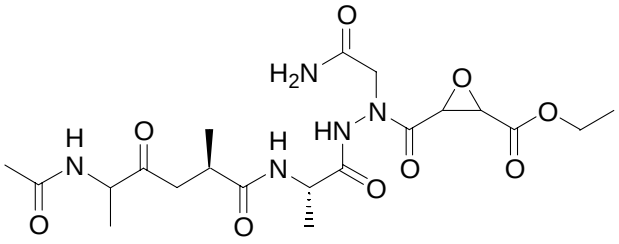
AEPI-3

AEPI-4 is R_1-Ala-Ala-AzaAsn-CH=CH-CON(CH_3)Bzl, for example, a compound of the structure:



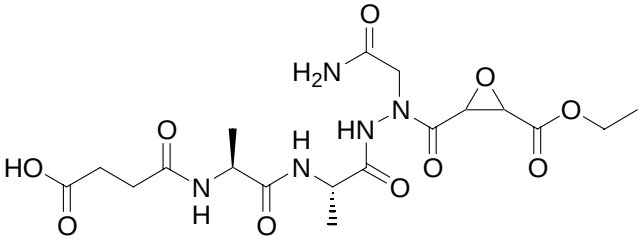
AEPI-4

AEPI-5 is N-acetyl-Ala-Ala-AzaAsn-(S,S)-EPCOOEt, a compound of the structure:



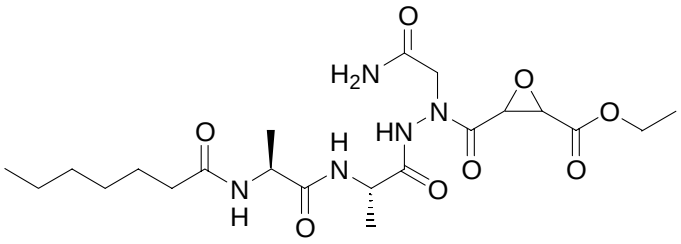
AEPI-5

AEPI-6 is N-succinyl-Ala-Ala-AzaAsn-(S,S)-EPCOOEt, a compound of the structure:



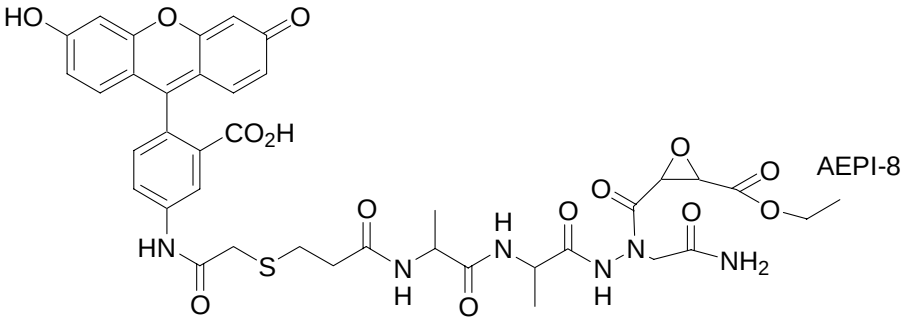
AEPI-6
MW:473.4345

AEPI-7 is N-hepatanoyl-Ala-Ala-AzaAsn-(S,S)-EPCOOEt, a compound of the structure:



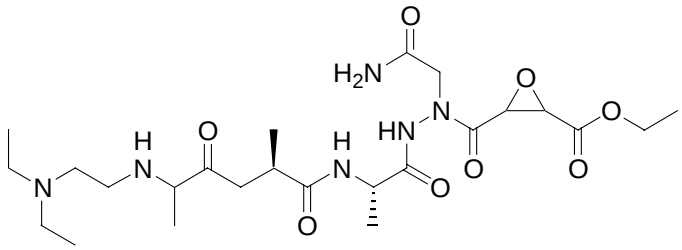
AEPI-7
MW:485.5313

AEPI-8 is 3-(N-carbamoylmethyl-N'-{fluorescein]-methylsulfanyl}-N-propionyl)-Ala-Ala-AzaAsn-(S,S)-EPCOOEt, a compound of the structure:



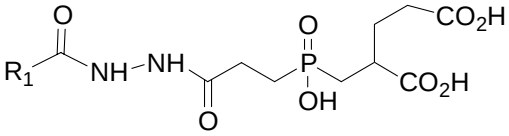
AEPI-8

AEPI-9 is N-triethylamino-Ala-(N-isopropanoyl-Ala-AzaAsn-(S,S)-EPCOOEt), a compound of the structure:



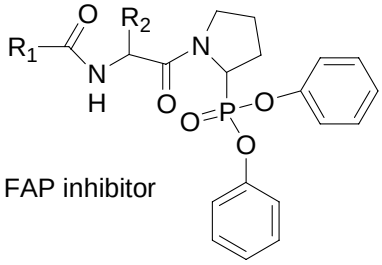
wherein R_1 is hydrogen, a hydrophilic group, a hydrophobic group, a photosensitizing agent, a label or an amino protecting group; Ep is epoxy; Et is ethyl; and Bzl is benzyl.

Inhibitors of PSMA that can be used in the invention include the following:



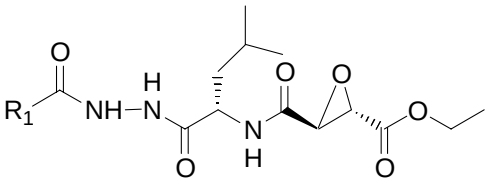
wherein R_1 is hydrogen, a hydrophilic group, a hydrophobic group, a photosensitizing agent, a label or an amino protecting group.

Inhibitors of fibroblast activation protein (FAP) that can be used in the invention include the following:



wherein R_1 is hydrogen, a hydrophilic group, a hydrophobic group, a photosensitizing agent, a label or an amino protecting group; and R_2 is hydrogen, hydroxymethylene (CH_2OH), lower alkyl (e.g., methyl, ethyl, propyl, isopropyl ($CH(CH_3)_2$), butyl, isobutyl), or benzyl.

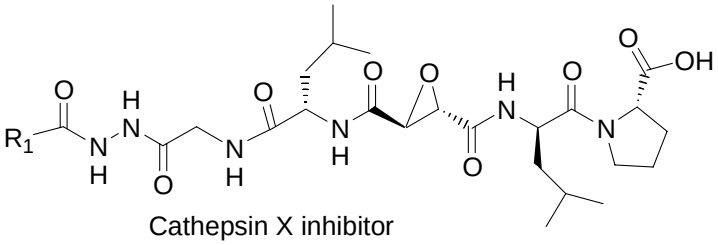
Cathepsin B inhibitors useful in the invention include the following:



Cathepsin B inhibitor

wherein R_1 is hydrogen, a hydrophilic group, a hydrophobic group, a photosensitizing agent, a label or an amino protecting group.

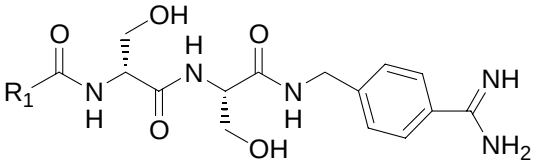
Cathepsin X inhibitors useful in the invention include the following:



Cathepsin X inhibitor

wherein R_1 is hydrogen, a hydrophilic group, a hydrophobic group, a photosensitizing agent, a label or an amino protecting group.

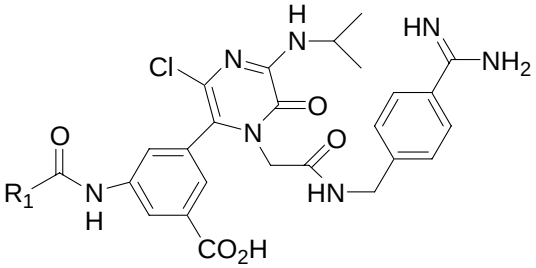
Urokinase-type plasminogen activator (uPA) inhibitors useful in the invention include the following:



uPA inhibitors

wherein R_1 is hydrogen, a hydrophilic group, a hydrophobic group, a photosensitizing agent, a label or an amino protecting group.

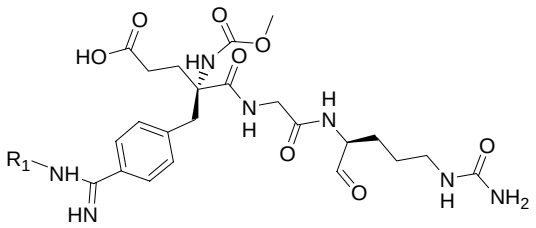
Tissue factor VIIa (TF VIIa) inhibitors useful in the invention include the following:



TF/VIIa inhibitor

wherein R_1 is hydrogen, a hydrophilic group, a hydrophobic group, a photosensitizing agent, a label or an amino protecting group.

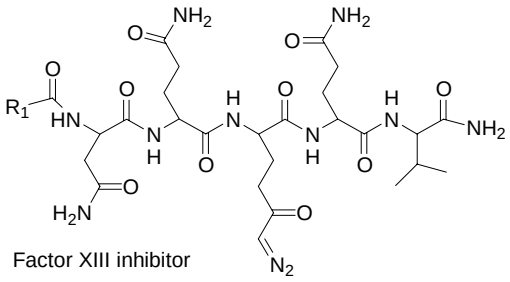
Matriptase inhibitors useful in the invention include the following.



Matriptase inhibitor

wherein R_1 is hydrogen, a hydrophilic group, a hydrophobic group, a photosensitizing agent, a label or an amino protecting group.

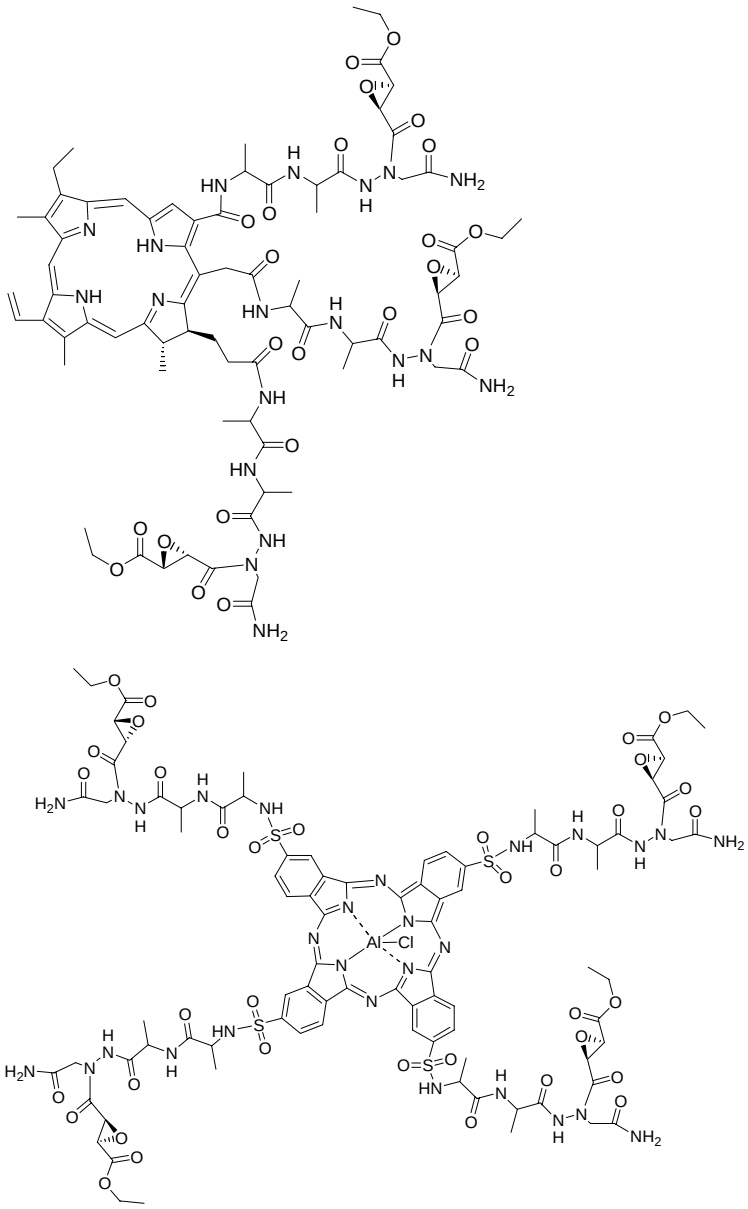
Factor XIII inhibitors useful in the invention include the following.



Factor XIII inhibitor

wherein R_1 is hydrogen, a hydrophilic group, a hydrophobic group, a photosensitizing agent, a label or an amino protecting group.

In some embodiments, the inhibitor is linked to a photosensitizing agent. While any available photosensitizing agent can be used (e.g., any of the photosensitizing agents contemplated for use with the present prodrugs), specific examples of photosensitizing agents include chlorin e6 and aluminum phthalocyanine tetrasulfonate (AlPcS4). Examples of inhibitors with chlorin e6 and AlPcS4 are shown below.

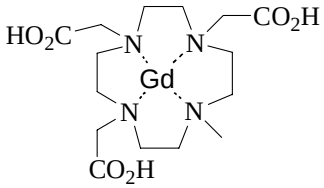


Hydrophilic R_1 groups of the invention can be sugars (monosaccharides and disaccharides), dicarboxylic acids (e.g., succinate, malate, fumarate, oxaloacetate, citrate, isocitrate), glycans, polyalkylene oxides, lower alkyl carboxylates, carboxyalkyls, carboxyalkylene carboxylates, and the like.

Hydrophobic R_1 groups of the invention can be alkyl, aryl, alkylene aryl, arylalkyl, hydrophobic amino protecting agents, beta-alanyl and related hydrophobic groups.

Any convenient amino protecting group available in the art can be used in the invention including, for example, carbobenzyloxy (Cbz), *tert*-butyloxycarbonyl (BOC), 9-fluorenylmethyloxycarbonyl (FMOC) and benzyl groups.

A variety of labels can be used with the inhibitors of the invention to generate imaging agents or reagents for detection of cancer. Such labels can be fluorophores, radioisotopes, metals, enzymes, enzyme substrates, luminescent moieties, and the like. One example of a label that may used is gadolinium or a gadolinium complex. For example, the following gadolinium complex can be used as a label:



In other embodiments, the legumain inhibitor can be cystatin, stefin, a peptide including the sequence Ala-Leu-β-Asn-Ala-Ala (SEQ ID NO:15) or an antibody that inhibits legumain activity.

Another aspect of the invention is a pharmaceutical composition that includes at least one of the prodrug compounds of the invention or at least one of the protease inhibitors of the invention and a pharmaceutically acceptable carrier. In some embodiments, the carrier is a liposome. Combinations of the present prodrug compounds and/or protease inhibitors can also be included in the compositions of the invention.

Another aspect of the invention is a method of detecting and treating cancer in a mammal, comprising administering to the mammal an inhibitor of the invention, wherein R_1 is a label, to detect whether the mammal has cancer and to detect which type of tumor specific protease is associated with the cancer, and administering a prodrug of the invention to treat the cancer, wherein the prodrug has a cleavage site fot the tumor specific protease associated with the cancer.

Another aspect of the invention is a method for treating a mammal having, or suspected of having cancer. The method includes administering to the mammal a prodrug compound and/or a protease inhibitor of the invention in amounts and at

intervals effective to prevent, reduce, or eliminate one or more of the symptoms associated with cancer. The cancer can be an invasive or metastatic cancer. The cancer can also be a tumor that is prone to angiogenesis. Cancers that can be treated by the invention include solid tumors and cancers as well as cancers associated with particular tissues, including breast cancer, colon cancer, lung cancer, prostate cancer, ovarian cancer, cancer of the central nervous system, carcinomas, leukemias, lymphomas, melanomas, fibrosarcomas, neuroblastoma, and the like. The cancer can, for example, be autoimmune deficiency syndrome-associated Kaposi's sarcoma, cancer of the adrenal cortex, cancer of the cervix, cancer of the endometrium, cancer of the esophagus, cancer of the head and neck, cancer of the liver, cancer of the pancreas, cancer of the prostate, cancer of the thymus, carcinoid tumors, chronic lymphocytic leukemia, Ewing's sarcoma, gestational trophoblastic tumors, hepatoblastoma, multiple myeloma, non-small cell lung cancer, retinoblastoma, or tumors in the ovaries.

The invention also provides a method for inhibiting cancer metastasis and/or tumor cell invasion in an animal, including administering a protease inhibitor compound and/or prodrug of the invention to the animal in amounts and at intervals effective to prevent, reduce, or eliminate cancer metastasis and/or tumor cell invasion.

The invention also provides a method for inhibiting cell migration in an animal that includes administering a protease inhibitor compound and/or prodrug of the invention to the animal in amounts and at intervals effective to prevent, reduce, or eliminate cancer cell migration.

The invention also provides a method of killing a cell in a tissue, including contacting the cell with a prodrug of the invention in amounts and at intervals effective to kill the cell, wherein the tissue includes cells that express legumain.

The invention also provides a method for treating cancer in animal that includes administering to the animal a protease inhibitor compound or a prodrug of the invention in amounts and at intervals effective to prevent, reduce, or eliminate one or more symptoms of cancer in the animal.

The invention also provides a method for inhibiting cancer metastasis in a tissue that includes contacting the tissue with a protease inhibitor compound or prodrug of the

invention in amounts and at intervals effective to prevent, reduce, or eliminate cancer metastasis.

The invention also provides a method for inhibiting cancer cell migration in a tissue that includes contacting the tissue with a protease inhibitor compound or prodrug of the invention in amounts and at intervals effective to prevent, reduce, or eliminate cancer cell migration.

The invention also provides a method for treating inflammation in an animal, which includes administering to the mammal a protease inhibitor compound or prodrug of the invention in amounts and at intervals effective to prevent, reduce, or eliminate one or more symptoms associated with inflammation.

The invention also provides a method for delivering a drug to a cell in a tumor microenvironment of a mammal, which includes administering to the mammal an effective amount of a prodrug of the invention.

The invention also provides a method for diagnosing cancer in a tissue that includes contacting the tissue with of an agent that specifically binds to a protease that is expressed in a tumor microenvironment, and detecting whether the agent binds to the tissue. The invention also provides a method for diagnosing cancer in an animal that includes administering to the animal an agent that specifically binds to a protease that is expressed in a tumor microenvironment, and detecting whether the agent accumulates in a tissue. For example, the protease can be selected from the group legumain, PSMA, FAP, Cathepsin B, Cathepsin X, uPA, TF VIIa, matriptase and Factor XIII. These methods can further include diagnosing the patient as having or not having cancer and monitoring the progression of a cancer.

The invention also provides a method for imaging a tissue that expresses a protease. For example, the protease can be selected from the group legumain, PSMA, FAP, Cathepsin B, Cathepsin X, uPA, TF VIIa, matriptase and Factor XII. The method includes contacting a test tissue suspected of expressing the protease with of an agent that specifically binds to the protease, and detecting whether the agent binds to the test tissue. The method can further include quantifying and comparing amounts of the agent bound to the test tissue with amounts of the agent bound to a control tissue that does

not express the protease. The agent can be protease inhibitor-imaging agent or a labeled antibody that specifically binds to the protease.

In another embodiment, the prodrug and inhibitor compounds of the invention can be used for the manufacture of a medicament useful for treating diseases such as cancer.

Targeting tumor-associated macrophages as a novel strategy against breast cancer

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Tumor-associated macrophages (TAMs) are associated with tumor progression and metastasis. Here, we demonstrate for the first time that legumain, a member of the asparaginyl endopeptidase family functioning as a stress protein, overexpressed by TAMs, provides an ideal target molecule. In fact, a legumain-based DNA vaccine served as a tool to prove this point, as it induced a robust $CD8^+$ T cell response against TAMs, which dramatically reduced their density in tumor tissues and resulted in a marked decrease in proangiogenic factors released by TAMs such as TGF-β, TNF-α, MMP-9, and VEGF. This, in turn, led to a suppression of both tumor angiogenesis and tumor growth and metastasis. Importantly, the success of this strategy was demonstrated in murine models of metastatic breast, colon, and non–small cell lung cancers, where 75% of vaccinated mice survived lethal tumor cell challenges and 62% were completely free of metastases. In conclusion, decreasing the number of TAMs in the tumor stroma effectively altered the tumor microenvironment involved in tumor angiogenesis and progression to markedly suppress tumor growth and metastasis. Gaining better insights into the mechanisms required for an effective intervention in tumor growth and metastasis may ultimately lead to new therapeutic targets and better anticancer strategies.

Introduction

A novel antitumor strategy is immunization against molecules overexpressed by tumor-associated macrophages (TAMs) and thereby remodel the tumor microenvironment that attracts these macrophages and mediates their function (1, 2). TAMs consist primarily of a polarized M2 ($F4/80^+/CD206^+$) macrophage population with little cytotoxicity for tumor cells because of their limited production of NO and proinflammatory cytokines (3). TAMs also possess poor antigen-presenting capability and effectively suppress T cell activation. In fact, these macrophages of M2 phenotype actually promote tumor cell proliferation and metastasis by secreting a wide range of growth and proangiogenic factors as well as metalloproteinases and by their involvement in signaling circuits that regulate the function of fibroblasts in the tumor stroma (4). In recent studies, anti-TAM effects induced by small molecule inhibitors contributed to tumor suppression (5, 6). For example, the antineoplastic agent Yondelis has a selective cytotoxic effect on TAMs, thereby significantly reducing their production of IL-6 and CCL2, which, in turn, contribute to growth suppression of inflammation-associated human tumors (7). Another such example is provided by a biphosphonate compound, zoledronic acid, that suppresses MMP-9 secretion by TAMs, thereby inhibiting tumor metalloproteinase activity and diminishing the association of VEGF with its tyrosine kinase receptors on proliferating endothelial cells (8). In a different experimental model, the chemokine CCL5 was shown to be key in the recruitment of TAMs, and an antagonist of this chemokine reduced the tumor infiltrate and slowed tumor growth (9). Hence, although the therapeutic targeting of TAMs is still in its infancy, initial clinical results are encouraging, as they suggest that targeting TAMs may complement more conventional cancer treatment regimens.

Legumain is a novel evolutionary offshoot of the C13 family of cysteine proteases (10). It is well conserved in plants and mammals, including humans. It was first identified in plants as a processing enzyme of storage proteins during seed germination (11, 12) and was subsequently identified in parasites and then in mammals (13). Legumain is a robust acidic cysteine endopeptidase with remarkably restricted specificity, absolutely requiring an asparagine at the P1 site of its substrate sequence (13). The selection of legumain as a target for tumor therapy is based on the fact that the gene encoding this asparaginyl endopeptidase was found to be highly upregulated in many murine and human tumor tissues (1, 14, 15) but absent or present only at very low levels in all normal tissues from which such tumors arise. Importantly, overexpression of legumain occurs under such stress conditions as tumor hypoxia, which leads to increased tumor progression, angiogenesis, and metastasis. In this regard, we recently discovered that legumain is heavily overexpressed by TAMs in murine breast tumor tissues by using gene expression profiling and immunohistochemistry. Importantly for our studies, TAMs have a particularly abundant expression in the tumor stroma (14) and express high levels of legumain in this tumor microenvironment. In contrast, classical macrophages of the M1 phenotype, which perform key immune-surveillance and antigen-presentation functions, do not express legumain. Consequently, targeting TAMs that overexpress legumain does not interfere with the biological functions of M1 macrophages, including cytotoxicity and antigen presentation (16–18).

Based on these findings, we hypothesized that targeting TAMs that overexpress legumain will reduce their density and thereby remodel the tumor microenvironment. This should lead to the downregulation of a wide variety of tumor growth factors, proangiogenic factors, and metalloproteinases released by these M2

Nonstandard abbreviations used: TAM, tumor-associated macrophage.

Conflict of interest: The authors have declared that no conflict of interest exists.

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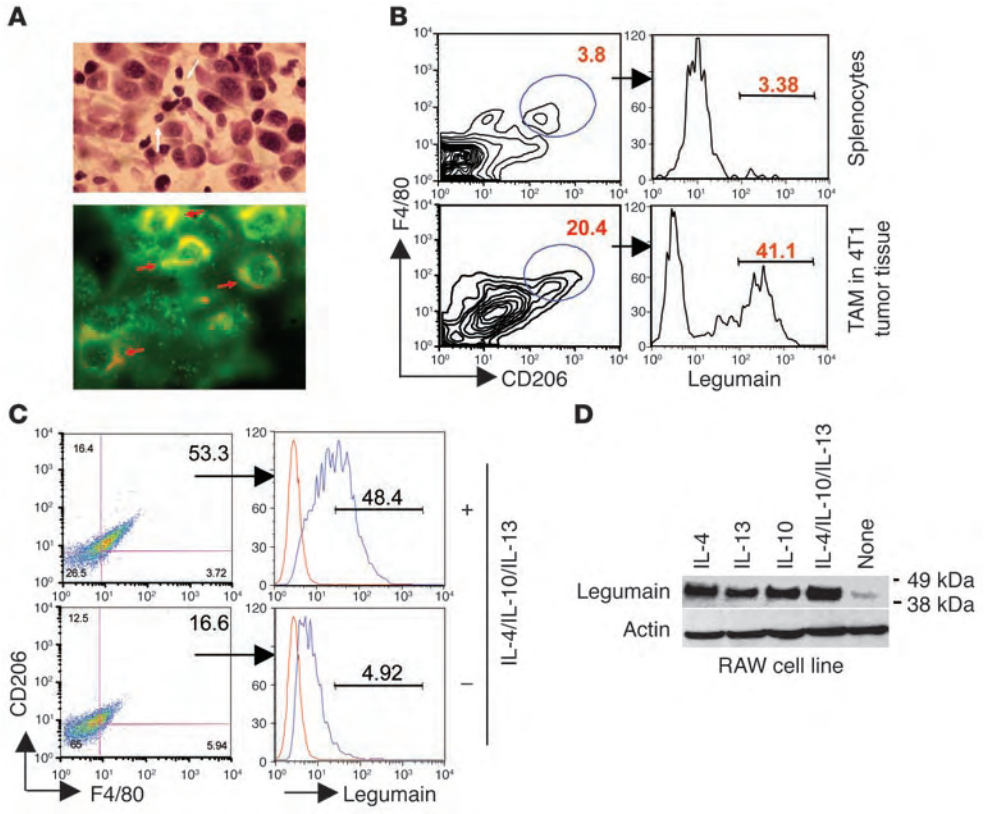

Figure 1
Legumain is highly expressed on TAMs in the tumor stroma. (**A**) Legumain expression on TAMs was clearly evident. Tumor-infiltrating macrophages were visualized by H&E staining, as indicated by arrows. Legumain expression is indicated by double staining with anti-legumain Ab (green) combined with anti-CD68$^+$ Ab (red). Magnification, ×350. (**B**) Increased legumain expression on TAMs was confirmed by flow cytometric analyses with double-positive populations of F4/80$^+$/CD206$^+$ M2 macrophages that were isolated from fresh tumor tissue. (**C**) Multiple-color flow cytometry demonstrated upregulation of the M2 macrophage marker CD206 on RAW cells after being cultured with IL-4, IL-10, and IL-13 (10 ng/ml). Furthermore, legumain was shown to be highly expressed on F4/80$^+$/CD206$^+$-positive RAW cells cultured with IL-4, IL-10, and IL-13 (upper panels) compared with wild-type RAW cells (lower panels). (**D**) Confirmation of legumain expression on RAW cells by Western blotting following stimulation with IL-4, IL-13, and IL-10, either singularly or combined.

macrophages and thereby decisively suppress angiogenesis of tumors as well as their growth and metastasis. To test our hypothesis, a legumain-based DNA vaccine served as a tool to eliminate TAMs in murine models of colon, breast, and lung tumor metastases.

Results

Legumain serves as a target to kill TAMs overexpressed during tumor progression. It is well known that TAMs play a key role in tumor progression and metastasis (5). Therefore, targeting of these M2 macrophages represents a novel antitumor strategy. We initially identified legumain as a significant marker molecule of TAMs, since it was highly overexpressed on these cells in the tumor microenvironment and stroma. To this end, we isolated TAMs from murine 4T1 breast tumor tissue and demonstrated by flow cytometry that legumain was highly overexpressed on CD206 and F4/80 double-positive M2 macrophages, especially when compared with normal M1 macrophages in the spleen (Figure 1B). This result was also confirmed by immunohistochemical analyses, indicating that TAMs could be visualized by H&E staining, and legumain overexpression was further indicated by double staining with anti-legumain Ab (Figure 1A, green) combined with anti-CD68 Ab (red). These data demonstrate that infiltrating TAMs are a disproportionally large cell subpopulation in 4T1 tumor tissue and that legumain is a potentially effective target for killing TAMs.

Induction of legumain expression on TAMs by Th2 cytokines. In order to determine whether legumain expression on TAMs was induced by such Th2 cytokines as IL-4, IL-10, and IL-13, we cocultured a murine macrophage cell line, RAW, with these cytokines. This resulted in a significant increase in F4/80$^+$/CD206$^+$ expression by these RAW cells, concurrent with an upregulation of legumain (Figure 1C). These results were confirmed by Western blotting (Figure 1D). However, we found no evidence for legumain expression by tumor cell lines when cultured with these same cytokines (data not shown). These findings suggest that Th2 cytokines such as IL-4, IL-10, and IL-13 are released by tumor and other tumor stromal cells and accumulate in the tumor microenvironment, where they could potentially induce the proliferation and transformation from M1 macrophages to a population with an M2 phenotype that overexpresses legumain.

Targeting of TAMs suppresses tumor progression. Growth and metastases of tumors are highly coordinated with the presence of TAMs, and therefore targeting of this macrophage subpopulation leads to suppression of tumor growth and metastases. To test this hypothesis, we generated an expression vector for a DNA vaccine encoding legumain. Figure 2A schematically depicts the vector construction map based on the pCMV/myc/cyto vector backbone. The gene encoding legumain was fused to the C terminus of mutant polyubiquitin (pLegumain) to improve antigen processing in the proteasome (19), and the entire fragment was then inserted between the PstI and NotI restriction sites, while protein expression was demonstrated by Western blotting. We further tested our hypothesis that reducing the number of TAMs by our legumain-based DNA vaccine can effectively inhibit spontaneous 4T1 breast cancer metastases or experimental metastases of either D121 non–small cell lung or CT26 colon carcinomas. Thus, in a prophylactic setting, C57BL/6J mice were immunized 3 times with either PBS, empty vector, or pLegumain carried by attenuated *Salmonella typhimurium*. One week after the last immunization, these mice were challenged i.v. with 2×10^5 D121

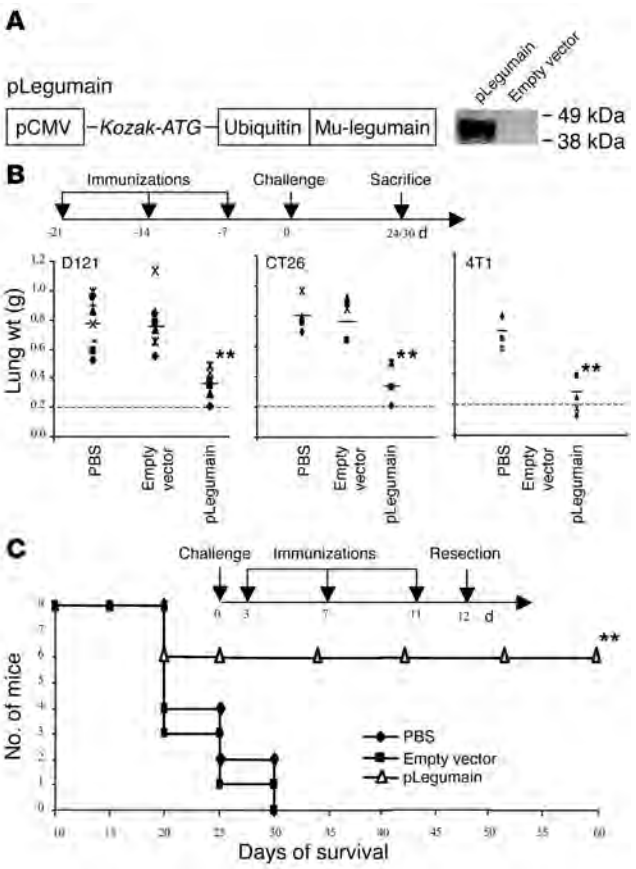

Figure 2
Targeting of legumain-expressing cells results in suppression of tumor progression. (**A**) Schematic of DNA vaccines constructed with the pCMV/myc/cyto vector backbone where the legumain gene was fused to the C terminal of mutant polyubiquitin. The entire fragment was inserted, and protein expression was demonstrated by Western blotting. Mu-legumain, murine legumain. (**B**) Prophylactic model: The vaccination schedule was designed for 3 immunizations at 1-week intervals followed by an i.v. challenge with 2×10^5 D121 non–small cell lung cancer cells and 5×10^4 CT26 colon cancer cells and mammary fat pad injection with 7×10^3 4T1 breast cancer cells. Lung weights were determined 24 days (D121 or CT26) or 30 days (4T1) after tumor cell challenge and analyzed in each group. Differences between the 2 control groups (PBS and/or empty vector) and the treatment group were statistically significant; $^{**}P < 0.005$. Pre-challenge lung weight, 0.2 g. (**C**) Therapeutic model: Groups of BALB/c mice ($n = 8$) were initially injected in the mammary fat pad with 7×10^3 4T1 breast cancer cells and thereafter vaccinated 3 times on days 3, 7, and 11 with PBS, empty vector, or the pLegumain vaccine, respectively, and primary tumors excised on day 12. Survival plots represent results for 8 mice in each of the treatment and control groups. The difference between the empty vector control group and the treatment group was statistically significant; $^{**}P < 0.005$.

non–small lung tumor cells, and 24 days thereafter experimental lung metastases were measured and analyzed. In the 2 control groups, the average lung weight was significantly greater than that of the vaccination group (Figure 2B). Similar results were obtained in the CT26 colon tumor model and 4T1 breast cancer model in syngeneic BALB/c mice (Figure 2B).

In a more demanding therapeutic setting, BALB/c mice were first challenged with 4T1 breast cancer cells and then immunized 3 times with the legumain-based DNA vaccine or an empty control vector. Twelve days after challenge with 4T1 tumor cells, the primary tumor was surgically excised, and the resulting lifespan curve indicated that 75% (6/8) of the mice immunized with pLegumain survived for 3 months. In contrast, mice in the control groups all died within 1 month (Figure 2C). These data indicate that the legumain-based DNA vaccine effectively suppresses tumor cell growth and metastases in mouse models of 4T1 breast cancer, D121 non–small cell lung cancer, and CT26 colon carcinoma. Combined with surgery, this vaccine could indeed significantly extend the life span of mice by inhibiting tumor cell metastases in these very challenging therapeutic mouse tumor models.

Targeting legumain induces a specific CD8⁺ CTL response, decreasing TAM populations in the tumor stroma. Immunization against legumain induced a specific T cell response against TAMs that highly express this asparaginyl endopeptidase. This was demonstrated by a ^{51}Cr release assay, in which splenocytes isolated from mice successfully immunized with this vaccine were effective in killing RAW macrophages, which expressed high levels of legumain after culture with cytokines IL-4, IL-10, and IL-13; however, these same splenocytes failed to induce cytotoxic killing of cells that lacked legumain expression (Figure 3A), indicating the specificity of this T cell response against legumain. Additionally, the same result was obtained using legumain-transfected cells as target cells in ^{51}Cr release assays (Supplemental Figure 1; available online with this article; doi:10.1172/JCI27648DS1). Furthermore, the results depicted in Figure 3B demonstrate a dramatic decrease in the F4/80⁺/CD206⁺ macrophage population after legumain-based DNA vaccine treatment. These data were also confirmed by immunohistochemical staining, as shown in Figure 3C.

MHC class I–restricted CD8⁺ CTLs are specifically active against TAMs. In gaining some insight into the immune mechanisms involved in the cytotoxic activity against TAMs, we found that DCs in Peyer's patches of successfully immunized mice were activated 3 days after vaccination with pLegumain, as indicated by the upregulated DC activation markers CD40, CD80, and MHC class I (Figure 4A). Furthermore, CD8⁺ T cell activation was found to be specific for legumain, as indicated by double staining for IFN-γ and CD8 on splenocytes obtained from successfully vaccinated mice (Figure 4B) and by the specific release of IFN-γ by activated T cells stimulated with legumain-positive cells (Figure 4C). In addition, in vivo immune depletion of CD4⁺ or CD8⁺ T cells revealed that only CD8⁺ T cells play a major role in the specific cytotoxic killing of TAMs, since only their depletion completely abrogated this killing effect. This specific cytotoxicity was MHC class I antigen restricted, as killing was specifically inhibited by anti–H-2D^d/H-2K^d antibodies (Figure 4D). Taken together, our results suggest that the legumain-based DNA vaccine first activated DCs in Peyer's patches, after which these cells presented legumain peptides through the MHC class I antigen pathway to the TCR on activated CD8⁺ T cells, resulting in a specific cytotoxic CD8⁺ T cell response abrogating TAMs.

Abrogation of TAMs in the tumor stroma reduces the release of tumor growth factors and proangiogenic factors as well as inhibiting tumor cell migration and metastases. TAMs can influence tumor metastasis in several ways, as they secrete a wide variety of tumor growth factors, proangiogenic factors, and tumor-associated enzymes that

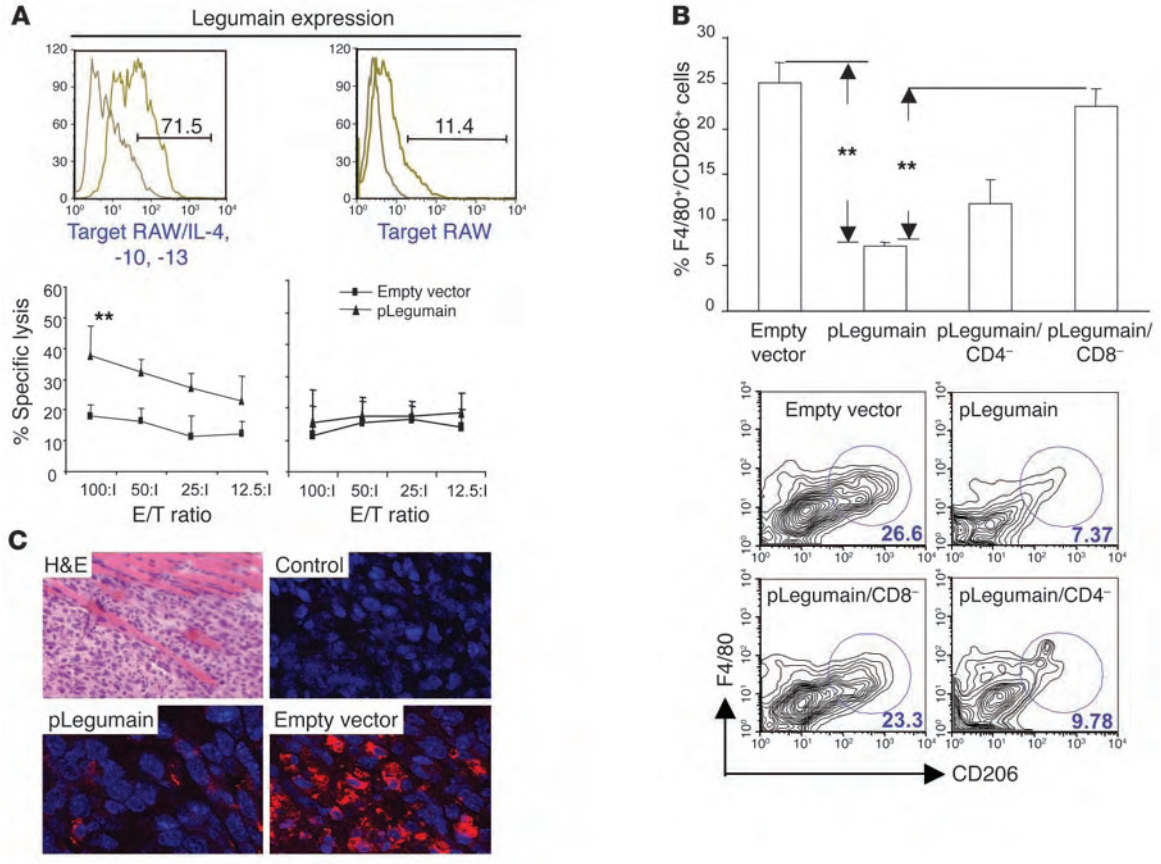

Figure 3

TAM population in the tumor stroma is decreased by CD8+-specific CTLs induced by the legumain-based DNA vaccine. (**A**) RAW macrophage cells, which highly express legumain after culturing with 10 ng/ml IL-4, IL-10, and IL-13, served as target cells in a 4-hour ^{51}Cr release assay. Splenocytes isolated from mice immunized with the pLegumain vaccine were shown to effectively kill RAW cells treated with these cytokines in vitro at different effector-to-target (E/T) cell ratios but failed to induce cytotoxic killing of unstimulated RAW cells lacking legumain expression. $^{**}P < 0.005$ compared with control groups. (**B**) The percentage of TAM populations with specific macrophage markers (CD206 and F4/80) in tumor tissue with or without vaccination was detected by flow cytometry. The percentage of TAM populations among tumor tissue cells isolated from mice treated with our DNA vaccine was shown to be reduced; however, there was no decrease in TAM populations isolated from mice treated with either empty vector or pLegumain following $CD8^+$ T cell depletion ($^{**}P < 0.005$). (**C**) The results of flow cytometry were confirmed by immunohistochemical staining evaluated by confocal microscopy. The population of TAMs in the tumor stroma was dramatically decreased after vaccination. 4T1 cancer cells are shown in blue and TAMs in red. Magnification, ×50 (H&E) and ×350 (control, empty vector, and pLegumain).

stimulate tumor angiogenesis and tumor growth and metastasis. In an effort to assess whether the elimination of TAMs actually reduced the release of some of these factors, serum and tumor tissue cells were collected from vaccinated mice and from suitable control animals. Freshly isolated tumor cells were cultured and their supernatants collected at 24 and 48 hours, respectively. An ELISA, performed to quantify TNF-α, VEGF, and TGF-β, indicated a significant reduction in TNF-α and VEGF in both tumor cell supernatants and mouse serum; however, TGF-β was significantly reduced only in serum but not in cell supernatants (Figure 5A). Immunohistological staining confirmed a decrease in the expression of these factors in tumor tissue (Figure 5B). In addition, a significantly decreased tumor cell migration was found when treatment and control groups were compared (Figure 5C) in a migration and invasion assay, which indicated that these characteristics of tumor cells changed after the vaccine-induced remodeling of the tumor microenvironment caused by the reduction in TAMs. The ability to form tumor metastases was confirmed in an in vivo experiment as the metastasis scores and lung weights — measured 24 days after primary tumor excision in a therapeutic setting, as described in Methods — decreased significantly when compared with the 2 control groups (Figure 5D).

Elimination of TAMs in the tumor stroma results in reduction of tumor angiogenesis. Importantly, there also was a marked antiangiogenic effect after elimination of TAMs in the tumor stroma, particularly since these M2 macrophages produced a wide range of pro-angiogenic factors. This was established by Matrigel assays that detected new blood vessel growth in vivo, an effect that could be quantified by staining the endothelium with FITC-labeled isolectin B4. These results clearly show that vessel growth was significantly reduced after vaccination with pLegumain (Figure 6B). It was also clearly indicated that much more blood vessels grew in Matrigel plugs in mice immunized with empty vector after evaluation by digital imaging and with Masson's trichrome staining (Figure 6A). Furthermore, an immunochemical histology assay

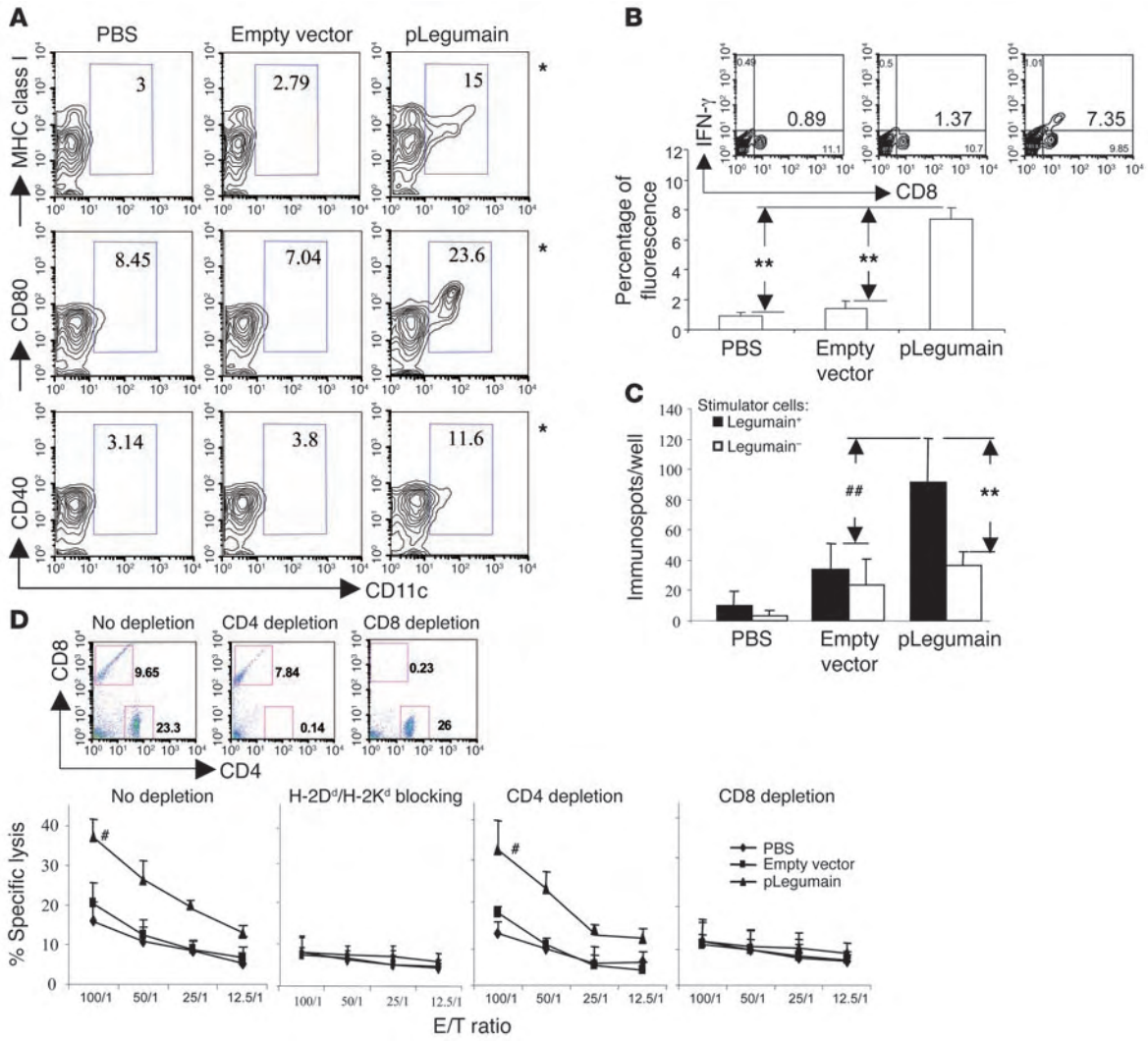

Figure 4

MHC class I antigen–restricted specific CD8+ T cell response against legumain-expressing cells. (**A**) DNA vaccination enhances expression of costimulatory molecules by DCs. Lymphocytes from Peyer's patches obtained 3 days after vaccination were stained with FITC-labeled anti-CD11cAb in combination with PE-conjugated anti-CD80, anti–MHC class I, or anti-CD40 Abs. *$P < 0.05$ compared with control groups. (**B**) Intracytoplasmic IFN-γ release of CD8+ T cells was measured by FACS analysis. **$P < 0.005$ compared with control groups. (**C**) Production of specific IFN-γ was verified at the single-cell level by ELISPOT. This is indicated for lymphocytes from immunized mice restimulated with either legumain+ 4T1 tumor tissue cells or legumain− 4T1 cells, as indicated by the number of immunospots formed per well. **$P < 0.005$ compared with treatment group without stimulation; ##$P < 0.005$ compared with control groups. (**D**) Splenocytes isolated from treated mice were effective in killing TAMs according to a ^{51}Cr release assay (#$P < 0.01$ compared with control groups). Inhibition experiments with Abs against H-2K^d/H-2D^d MHC class I antigens showed that T cell–mediated tumor cell lysis was MHC class I antigen restricted. Furthermore, in vivo depletions of CD4+ or CD8+ T cells indicated that lymphocytes isolated from vaccinated mice, which were thereafter depleted of CD8+ T cells, failed to induce cytotoxic killing of target cells. However, depletion of CD4+ T cells did not abrogate cytotoxic killing of these same target cells. #$P < 0.01$ compared with PBS or empty vector group.

was performed to assess the type of cells that actually migrated into the Matrigel plugs. The confocal images taken indicated that endothelial cells expressing CD31+ or macrophages expressing CD68+ grew or migrated into Matrigel plugs to a considerably greater extent in the empty vector control group than in the vaccine treatment group (Figure 6C).

Discussion

This study establishes the new paradigm whereby a reduction in the density of TAMs in the tumor stroma decreases the release of factors potentiating tumor growth and angiogenesis. This, in turn, remodels the tumor microenvironment so as to markedly suppress tumor cell proliferation, vascularization, and metastasis. However, targeting TAMs in the tumor stroma raises the concern that their abrogation could interfere with the normal immunological functions of these important components of the innate immune system. We addressed this question in view of the fact that circulating monocytes are versatile precursors with the ability to differentiate into the various forms of specialized macrophages (3). In fact, the cytokine milieu profoundly affects the differentiation and function of tissue macrophages, and their functional polarization has been defined (3, 20, 21). Thus, macrophages

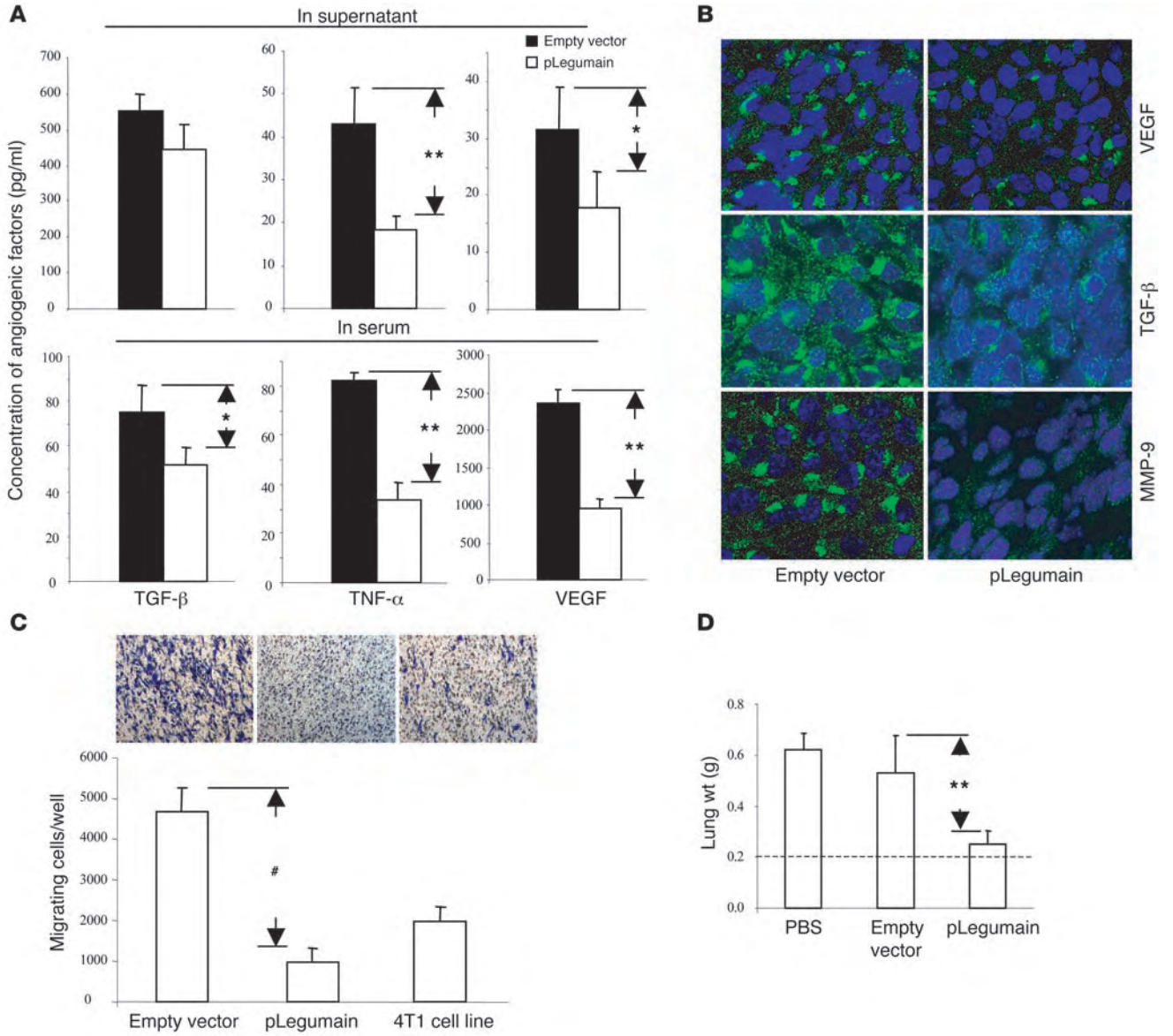

Figure 5

Abrogation of TAMs results in decreases of growth factor release, tumor cell migration, and metastases. (**A**) The vaccine decreased the release of growth factors by TAMs. 4T1 breast tumor tissue and mouse serum were harvested 12 days after vaccinations and tumor cell challenge. After 24 or 48 hours culturing, the supernatants of tumor tissue cells were harvested, and the concentrations of TGF-β, TNF-α, and VEGF in serum or supernatants measured by ELISA. There were significant differences between the treatment and control groups. $^{*}P < 0.01$; $^{**}P < 0.005$. (**B**) Immunohistochemical staining was performed to determine expression of these growth factors in the tumor microenvironment. In the vaccine treatment groups, VEGF, TGF-β, and MMP-9 release was decreased after a reduction in TAMs, compared with the empty vector groups. The growth factors are shown in green and 4T1 breast cancer cells in blue. (**C**) A Transwell migration assay was performed to determine tumor cell migration after vaccination. The number of migrating cells isolated from 4T1 tumor tissue was markedly reduced after vaccination. $^{\#}P < 0.001$ compared with the empty vector group. (**D**) In vivo experiments were performed to determine the ability of mice to form 4T1 tumor metastases. The mice were treated with the vaccine within the therapeutic setting as described above. Tumor metastasis scores and lung weights were measured 25 days after primary tumor excision. The metastasis scores are expressed as the percentage of lung surface covered by fused metastatic foci; 0: none; 1: <5%; 2: 5– 50%; 3: >50%. Scores for $n = 8$ mice/group were: PBS, 3, 3, 3, 3, 3, 3, 2, 2; empty vector, 3, 3, 3, 3, 3, 3, 3, 2; pLegumain, 2, 2, 1 0, 0, 0, 0, 0. Differences in lung weights between the group of mice treated with vaccine and all control groups were statistically significant ($^{**}P < 0.005$). Magnification, ×350 (**B**), ×50 (**C**).

activated by bacterial products and Th1 cytokines are regarded as being of the M1 phenotype, i.e., classically activated macrophages with high bactericidal activity and cytotoxic function against tumor cells. However, macrophages activated by such Th2 cytokines as IL-4 and IL-13 or immunosuppressors such as vitamin D3 and IL-10 are classified as macrophages with an M2 phenotype: low cytotoxic functions but high tissue-remodeling activity. Whereas M1 cells have immunostimulatory properties and

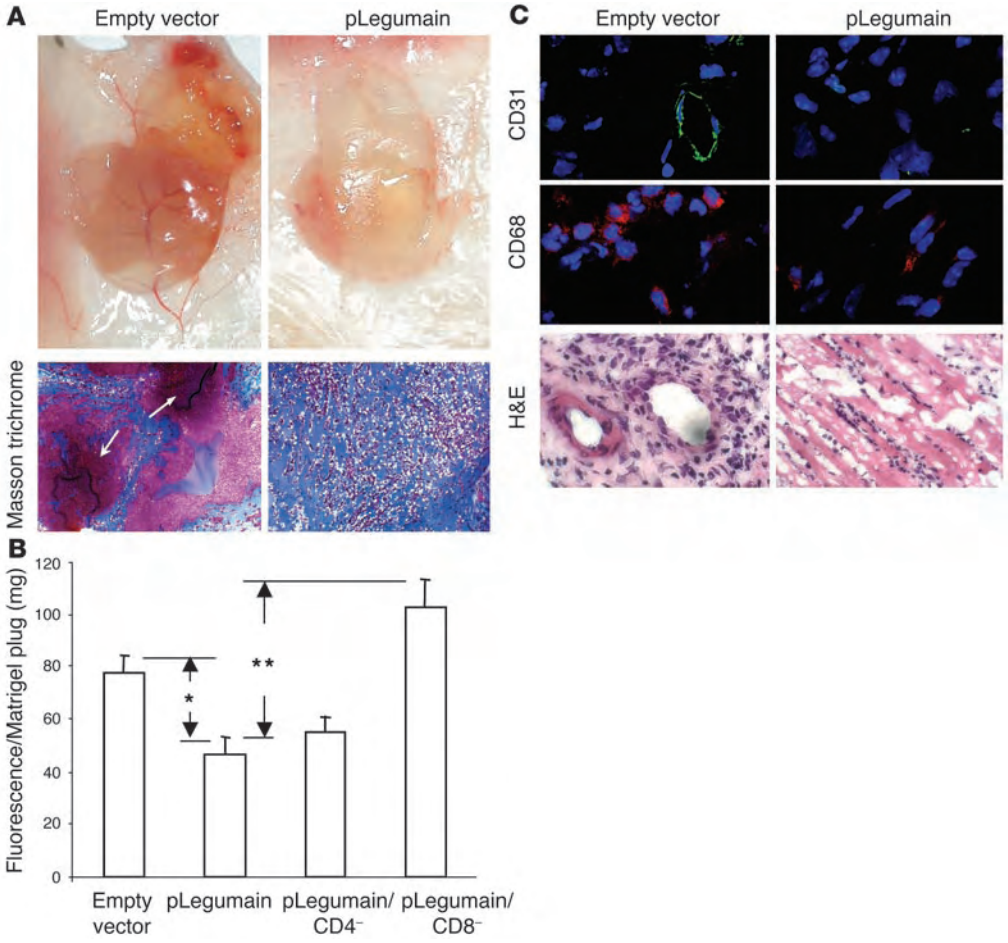

Figure 6

Elimination of TAMs results in a reduction in tumor angiogenesis. Suppression of VEGF-induced angiogenesis: BALB/c mice were vaccinated with empty vector, pLegumain, or pLegumain after either $CD8^+$ or $CD4^+$ T cell depletion in vivo. One week after the last vaccination, Matrigel was implanted s.c. into the midline of the abdomen of mice. Vascularization was induced by VEGF or bFGF. (**A**) The images were taken by a digital camera 6 days after Matrigel plug implantation. Additionally, the section of Matrigel plugs stained with Masson's trichrome indicate blood vessel growth in Matrigel plugs, as indicated by arrows. Magnification, ×50. (**B**) Quantification of vessel growth was performed after in vivo staining of endothelium with FITC-labeled isolectin B4 and evaluation by fluorimetry. There was a decrease in the VEGF-induced neovasculature only after vaccination with the vector encoding legumain but not after vaccination with the empty vector or with pLegumain after depletion of $CD8^+$ T cells. $^{**}P < 0.005$, $^{*}P < 0.01$ compared with the legumain treatment group. (**C**) Immunohistochemical staining was performed and evaluated by confocal microscopy. The cross-sections of Matrigel plugs were stained to determine the cell type that grew in or migrated into these plugs. The images indicate that endothelial cells with the CD31 marker or macrophages with the CD68 marker grew in or migrated into Matrigel plugs as indicated (magnification, ×350). H&E staining served as a control (magnification ×50).

defend the host against pathogenic infections, M2 cells attenuate acute inflammatory reactions, potently scavenge cellular debris, and secrete a variety of pro-growth and angiogenic factors essential for the repair of injured tissues. In addition, macrophages derived from healthy or inflamed tissue are capable of lysing tumor cells, expressing immunostimulatory cytokines, and presenting tumor-associated antigens to stimulate the proliferation and antitumor functions of T and NK cells. However, M2 macrophages, such as TAMs, show reduced levels of these activities. This may be the result of their exposure to tumor-derived antiinflammatory molecules such as IL-4, IL-10, TGF-β1, and prostaglandin E_2 (22, 23). Indeed, this finding prompted Mantovani and colleagues to suggest that exposure to IL-4 and IL-10 may induce monocytes in tumors to develop into polarized type II or M2 macrophages (24). To the extent that they have been investigated thus far, differentiated mature TAMs have a phenotype and function similar to those of type II macrophages (6). Therefore, cytokines present in the tumor microenvironment have the potential to promote and orient the differentiation of recruited mononuclear phagocytes (25). Indeed, a growing body of evidence indicates that TAMs are skewed toward M2 macrophages in the tumor microenvironment and produce a variety of pro-tumor growth and angiogenic factors as well as immunosuppressive molecules (1, 6, 26, 27). Thus, the presence of TAMs at the tumor site and the continuous expression and release of their products may favor, rather than antagonize, tumor progression and metastasis.

In our study we demonstrated that TAMs express abundant levels of CD206, a mannose receptor that is upregulated on M2 macrophages following exposure to IL-4 and IL-13 (27–30). We also established simultaneously that this population of macrophages expressed high levels of legumain. Importantly, we found that Th2 cytokines IL-4, IL-10, and IL-13 could upregulate the expression of CD206 and legumain on the macrophage cell line RAW. This finding can best be understood when one considers that macrophages are derived from peripheral blood and differentiate into M2 macrophages once they are recruited into tumor sites where IL-4, IL-13, and IL-10 are released by tumor cells and tumor stromal cells (3, 19, 29, 31). Thus, targeting of M2 macrophages expressing legumain not only benefits suppression of tumor growth and metastases but also maintains the normal functions of macrophages with the M1 phenotype.

The relationship between infiltration by TAMs and prognosis in tumor patients has also been indicated by several studies (1, 5, 32, 33), which concluded that the greater the macrophage infiltration, the worse the prognosis. Several lines of evidence indicate that a

symbiotic relationship exists in the tumor stroma between cancer cells and TAMs, whereby cancer cells attract TAMs and sustain their survival, while TAMs respond to tumor-derived molecules by producing important growth factors and extracellular matrix enzymes, which, in turn, stimulate tumor proliferation, angiogenesis, and invasion of surrounding tissues (18, 22, 26, 34). Thus, the attenuation of TAMs in the tumor environment can serve as an effective strategy to remodel the tumor stroma and to alter the tumor microenvironment (35).

In our study, a DNA construct encoding legumain evoked a robust CTL response against this asparaginyl endopeptidase, which functions as a stress protein that is highly overexpressed by TAMs. This immune response was shown to be MHC class I antigen restricted and $CD8^+$ T cell specific. Importantly, our data also demonstrated that after immunization with the legumain-based DNA vaccines, the density of $F4/80^+/CD206^+$ macrophages, i.e., TAMs, decreased dramatically. Furthermore, a variety of factors such as VEGF, MMP-9, and TGF-β that are released by TAMs were shown to be at low levels in both the supernatant of cultured tumor cells and mouse serum. Thus, it is well known that VEGF and metalloproteinase MMP-9 play important roles during the formation of the tumor vasculature and initiation of tumor angiogenesis. TAMs are important in this regard, since they produce both VEGF and MMP-9 (36). Progressively intensifying angiogenesis is associated with the upregulated expression of VEGF (37) and extracellular proteases, such as MMP-9 (8, 36), whereas TGF-β is known as an important growth factor involved in the migration of tumor cells toward blood vessels. In fact, TGF-β can provide proliferative and antiapoptotic signals to tumor cells as well as activate urokinase-type plasminogen activators (uPAs), which might contribute to the extracellular matrix breakdown that is required for vascular invasion to occur (38). Significantly, our data demonstrate that once TAMs were abrogated in the tumor tissue by specific $CD8^+$ CTLs, the tumor cells changed their character by becoming less malignant and less invasive. Also, the formation of a neovasculature in tumor tissues was reduced, since all of the factors released by TAMs that contribute to tumor angiogenesis were drastically reduced. Additionally, TAMs were reported to be involved in immune suppression and tolerance in the tumor microenvironment (39). It is also well known that TAMs may inhibit T cell responses by inducing apoptosis of activated T cells via upregulation of NO, PGs, TNF-α release, and arginase activity (40, 41). These may be some of the reasons for tumor immune tolerance, a notion also supported by our data. After abrogation of TAMs, the specific CD8 T cell activity was markedly upregulated, further supporting the contention that our anti-TAM approach could be a good strategy to break immune tolerance against tumors.

Furthermore, our hypothesis that a therapeutic approach using a legumain-based DNA vaccine to target TAMs holds much promise was strongly supported by data obtained in 3 tumor metastasis models used in our study. Thus, in the 4T1 spontaneous mouse breast carcinoma metastasis model, a significant increase in life span was obtained, as 75% (6/8) mice survived up to 3 months after 4T1 tumor cell inoculation into the mammary gland, once surgical resection of the primary tumor was followed by treatment with the legumain-based DNA vaccine. It was even more impressive that 62% (5/8) mice revealed no lung metastases at all. Similar results were obtained in prophylactic settings in the other 2 tumor models, i.e., D121 non–small cell lung carcinoma and CT26 colon carcinoma. These additional confirmatory data strengthen our contention that targeting of TAMs to remodel the tumor microenvironment might be a universal antitumor strategy for suppressing tumor cell invasion and metastases by reducing the concentration of factors released by TAMs that otherwise promote tumor growth and metastasis.

In summary, we critically evaluated the antitumor efficacy of targeting TAMs via the induction of a specific $CD8^+$ T cell response against legumain, which we identified for the first time as being a highly overexpressed target molecule on TAMs. In these experiments, we demonstrated that abrogation of TAMs in tumor tissues effectively decreased the release of several pro-tumor growth and angiogenic factors. It is likely that such an antitumor strategy could be widely applicable and relevant for possible clinical applications.

Methods

Animals, bacterial strains, and cell lines. Female BALB/c and C57BL/6 mice, 6–8 weeks of age, were purchased from The Scripps Research Institute Rodent Breeding Facility. The double-attenuated *S. typhimurium* strain RE88 (*aroA*$^-$;*dam*$^-$) was obtained from Remedyne Corp. The murine CT26 colon cancer cell line was kindly provided by I.J. Fidler (MD Anderson Cancer Center, Houston, Texas, USA), and the murine D121 non–small cell lung carcinoma cells were a gift from L. Eisenbach (Weizmann Institute of Science, Rehovot, Israel). The murine 4T1 breast carcinoma cells were kindly provided by Suzanne Ostrand-Rosenberg (University of Maryland, College Park, Maryland, USA).

Immunohistochemical analyses. These were performed on 4T1 tumor tissues and Matrigel plug sections. Legumain expression of macrophages was identified on 4T1 tumor tissue sections with biotinylated rat anti-mouse CD68 mAb (BD Biosciences — Pharmingen), with GFP-conjugated streptavidin being the secondary reporter reagent. Rabbit anti-legumain antiserum was prepared by immunization with purified human legumain produced in *Escherichia coli*. (10) The reaction was visualized with Texas red–conjugated streptavidin. Additionally, 4T1 tumor tissue sections and Matrigel plug sections were fixed and stained with MMP-9, VEGF, TGF-β, and F4/80 antibodies (eBioscience and Santa Cruz Biotechnology Inc.) in 4T1 tumor tissue section, while CD68 and CD31 Abs (BD Biosciences — Pharmingen) were used in Matrigel plug sections. All tissue sections were visualized with Texas red– or GFP-conjugated streptavidin as the secondary reporting reagent, and the slides were analyzed with laser scanning by confocal microscopy (Bio-Rad). All the images were captured by a SPOT Cooled Color Digital Camera System (Diagnostic Instruments Inc.).

Vector construction, protein expression, and transformation of S. typhimurium with DNA vaccine plasmids. Two constructs were made based on the vector pCMV (Invitrogen). The pLegumain construct consisted of polyubiquitinated, full-length murine legumain. The empty vector construct served as a control. Protein expression of legumain was demonstrated by Western blotting with a polyclonal rabbit anti-murine legumain Ab as well as anti-murine β-actin Ab (Santa Cruz Biotechnology Inc.) as a loading control. The specific protein was detected with a goat anti-rabbit HRP-conjugated IgG Ab (Bio-Rad). Attenuated *S. typhimurium* (*aroA*$^-$;*dam*$^-$) were transduced with DNA vaccine plasmids by electroporation as described in our previous publications (42, 43).

Immunization and tumor cell challenge. For the prophylactic model, BALB/c or C57BL/6 mice were each divided into 3 experimental groups ($n = 8$) and immunized with PBS, empty vector, or pLegumain. All mice were challenged by i.v. injection with 5×10^4 CT26 cells (BALB/c) or 2×10^5 D121 cells (C57BL/6) or injected in the mammary gland fat pad with 7×10^3 4T1 cells (BALB/c), 1 week after the last immunization, to induce either experimental or spontaneous pulmonary metastases. The lung weights

in experimental or control groups were determined 24 days after tumor cell challenge. For the therapeutic model, BALB/c mice were divided into 3 experimental groups (n = 8) and first injected in the fat pad with 7×10^3 4T1 cells on day 0 and then immunized 3 times with DNA vaccine starting on days 3, 7, and 11, and primary tumor was excised on day 12. The experiment was terminated 24 days after primary tumor excision to determine lung weights and metastasis scores or mouse survival rates.

In vivo depletion of CD4⁺ or CD8⁺ T cells, cytotoxicity, and ELISPOT assays. Analysis of the depletion of $CD4^+$ or $CD8^+$ T cells in vivo was performed as previously described (44). Cytotoxicity was measured and calculated by a standard ^{51}Cr release assay as previously reported (45). ELISPOT assays were performed with an ELISPOT kit (BD Biosciences — Pharmingen) according to instructions provided by the manufacturer.

In vivo Matrigel angiogenesis assay. Matrigel was used for evaluating the suppression of angiogenesis after vaccination. Briefly, BALB/c mice were injected s.c. 2 weeks after the last vaccination, in the sternal region, with Growth Factor Reduced BD Matrigel (BD Biosciences) containing bFGF-2 (200 ng/plug) and 4T1 tumor cells (5×10^3/plug) that were previously irradiated with 1,000 Gy. The endothelium was stained 6 days after Matrigel implantation by i.v. injection with *Bandeiraea simplicifolica* lectin I (isolectin B4), conjugated with fluorescein (Vector Laboratories). This was done along with staining the endothelium of control animals, and 30 minutes later, mice were sacrificed, Matrigel plugs extracted, and fluorescence evaluated by fluorimetry. Additionally, the Matrigel plugs were removed 6 days after Matrigel implantation, fixed in Bouin's solution for 24 hours, and then embedded in paraffin. All tissues were sectioned, mounted onto slides, and stained with Masson's trichrome. All of the images were captured by a SPOT cooled color digital camera system (Diagnostic Instruments Inc.)

Flow cytometry. DC cell markers were determined by staining freshly isolated lymphocytes from successfully vaccinated mice and control mice with PE-labeled anti-CD11c Ab in combination with FITC-conjugated anti-CD40, anti-CD80 Ab, and Abs against MHC class II antigen. Macrophages bearing high levels of $CD206^+$ and $F4/80^+$ were quantified by 2-color flow analysis. Tumor cells were isolated from successfully vaccinated BALB/c mice and then stained with anti-CD206 Ab conjugated with PE (Cell Sciences), anti-F4/80 Ab conjugated with APC, and anti-legumain Ab conjugated with FITC, followed by FACS analyses. All antibodies were purchased from BD Biosciences — Pharmingen. IFN-γ release at the intracellular level was determined in lymphocytes of Peyer's patches obtained 3 days after one-time immunization and stained with APC-conjugated anti-CD8 Ab. Cell were fixed, permeabilized, and subsequently stained with PE-labeled anti–IFN-γ Ab to detect intracellular expression of IFN-γ.

Migration assay. Cell migration assays were performed using modified Boyden chambers (Transwell; Corning Inc.). Transwell migration assays were performed with tumor cells harvested from tumor tissue of either vaccine-treated or control groups of mice. After 4 hours culture, the cells on the lower surface of wells were fixed with 1% paraformaldehyde, stained with 1% crystal violet, and counted (46).

Statistics. The statistical significance of differential findings between experimental groups and controls was determined by Student's t test. Findings were regarding as significant if 2-tailed P values were less than 0.05. Kaplan-Meier analysis was used to evaluate the survival of mice.

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Synthesis of the next-generation therapeutic antibodies that combine cell targeting and antibody-catalyzed prodrug activation

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An obstacle in the utilization of catalytic Abs for selective prodrug activation in cancer therapy has been systemic tumor targeting. Here we report the generation of catalytic Abs that effectively target tumor cells with undiminished prodrug activation capability. Ab conjugates were prepared by covalent conjugation of an integrin $\alpha_v\beta_3$-targeting antagonist to catalytic Ab 38C2 through either sulfide groups of cysteine residues generated by reduction of the disulfide bridges in the hinge region or surface lysine residues not involved in the catalytic activity. Using flow cytometry, the Ab conjugates were shown to bind efficiently to integrin $\alpha_v\beta_3$-expressing human breast cancer cells. The Ab conjugates also retained the *retro*-aldol activity of their parental catalytic Ab 38C2, as measured by methodol and doxorubicin (dox) prodrug activation. Complementing these Ab conjugates, an evolved set of dox prodrugs was designed and synthesized. Dox prodrugs that showed higher stability and lower toxicity were evaluated both in the presence and absence of the integrin $\alpha_v\beta_3$-targeting 38C2 conjugates for cell-killing efficacy by using human breast cancer cells. Our study reveals that cell targeting and prodrug activation capabilities can be efficiently combined for selective chemotherapy with novel dox prodrugs.

aldolase Ab | Ab conjugate | doxorubicin | integrin $\alpha_v\beta_3$

Monoclonal Abs with aldolase activity have emerged as highly efficient catalysts for a number of chemical transformations, particularly aldol and *retro*-aldol reactions (1). The excellent *retro*-aldolase activity (2, 3) of Abs 38C2 (4) and 93F3 (5) has allowed us to design, synthesize, and evaluate prodrugs of various chemotherapeutic agents that can be activated by *retro*-aldol reactions (6–10). In a syngeneic mouse model of neuroblastoma, systemic administration of an etoposide prodrug and intratumor injection of Ab 38C2 inhibited tumor growth (11). However, to evaluate the utility of these Abs for selective chemotherapy in a systemic setting, it will be necessary to go one step further and equip the Ab with a tumor-recognition device to target the catalytic Ab to the malignant cells. In previous studies, known as Ab-directed enzyme prodrug therapy or the Ab-directed abzyme prodrug therapy approach (12, 13), enzymes or catalytic Abs were directed to tumor cells by chemical conjugation or recombinant fusion to targeting Abs. As a potentially more efficient and chemically defined alternative, we have used a strategy in which the catalytic Ab is conjugated to a targeting device located outside the combining site, thereby leaving the active site available for the prodrug activation. Here we report chemical constructions in which Ab 38C2 is conjugated to a synthetic small-molecule-targeting device that mediates binding of the Ab to integrin $\alpha_v\beta_3$, a tumor and tumor vasculature cell-surface receptor (14). The conjugate of Ab 38C2 to an integrin $\alpha_v\beta_3$-binding synthetic small molecule is expected to selectively localize the Ab to the tumor and/or the tumor vasculature and trigger prodrug activation at that site. Complementing these efforts, we also describe the design, synthesis, and evaluation of a set of doxorubicin (dox) prodrugs that, when tested with an integrin $\alpha_v\beta_3$-expressing human breast cancer cell line, are less toxic than previously reported prodoxorubicins (prodoxs) and are activated more efficiently by Ab 38C2. Collectively, our studies open the way for the construction of a new class of therapeutic Abs that contain both targeting and drug activating moieties.

Results and Discussion

$\alpha_v\beta_3$ Integrin-Targeting Catalytic Ab Conjugates. Earlier, we prepared numerous Ab 38C2 arginine-glycine-aspartic acid peptidomimetics, including compound **1**, en route to the corresponding diketone derivatives that were used for the construction of noncatalytic 38C2 constructs (15–18). The central idea in these constructs was to conjugate a linker to a targeting device such that the linker was covalently attached to the Ab-combining site, and the targeting functionality extended beyond the surface of the Ab. In these conjugates, the diketone or the vinylketone compounds reacted in the Ab-binding sites through the reactive lysine residues to form an enaminone or Michael-type adduct, respectively, thereby displaying the conjugated peptidomimetics. These Abs bound to cells that expressed $\alpha_v\beta_3$ integrin, including human breast cancer cells MDA-MB-231, human Kaposi's sarcoma, and human melanoma. Thus, this is a convenient way to covalently attach organic ligands to Abs, but in the process, the catalytic activity of the Ab is destroyed. Although such constructs may be very useful in that they endow organic compounds with the relatively long half-lives and/or effector functions of Abs, they do not take advantage of the Ab's catalytic potential. We anticipated that a conjugate of **1** outside the active site of 38C2 would also bind integrin $\alpha_v\beta_3$-expressing cells and possibly retain the catalytic activity of the Ab. Therefore, starting from amine **1**, compounds **2** and **3** were prepared and conjugated to 38C2, affording 38C2-**2** and 38C2-**3** conjugates (Fig. 1).

In 38C2-**2**, compound **2** was conjugated through the reduced sulfide bonds in the Ab hinge region, whereas the activated ester **3** reacted to surface lysine residues of the Ab in 38C2-**3**.¶ The formation of 38C2-**2** and 38C2-**3** conjugates was confirmed by

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The authors declare no conflict of interest.

Abbreviations: dox, doxorubicin; prodox, prodoxorubicin; MVK, methyl vinyl ketone.

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¶An analogous 38C2–polymer conjugate was also prepared (see ref. 19).

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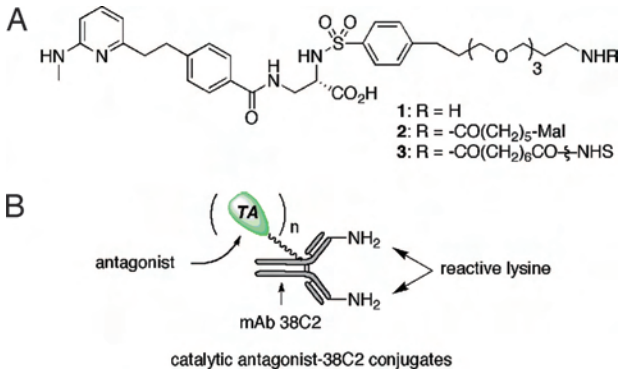


Fig. 1. Production of catalytic Ab conjugates. (*A*) Structure of small-molecule arginine-glycine-aspartic acid peptidomimetic antagonists equipped with a linker. (*B*) Schematic drawing of a catalytic cell-targeting antagonist–38C2 conjugate for targeting cells that express integrin $\alpha_v\beta_3$. Mal, maleimide; NHS, *N*-hydroxysuccinimide.

mass spectral analysis as well as determining their binding to MDA-MB-231 cells. As shown in Fig. 2, the mass spectra (MALDI-MS) of 38C2-**2** and 38C2-**3** recorded an increase of 1,761 and 1,049 mass units, respectively, suggesting that on average 2.0 and 1.3 molecules of **2** and **3** were attached to 38C2 in 38C2-**2** and 38C2-**3** conjugates. Next, the binding of conjugates 38C2-**2** and 38C2-**3** to MDA-MB-231 was determined as described (17). For comparison, we used the previously reported 38C2 conjugate (17) (prepared from 38C2 and the diketone derivative of **1**) and 38C2 alone as the positive and negative controls, respectively. As shown in Fig. 2*B*, the 38C2-**2** and 38C2-**3** conjugates and the positive control showed efficient binding to MDA-MB-231 cells. In contrast, 38C2 alone did not show any binding to these cells.

Once binding of the 38C2 conjugates to the cells was verified,

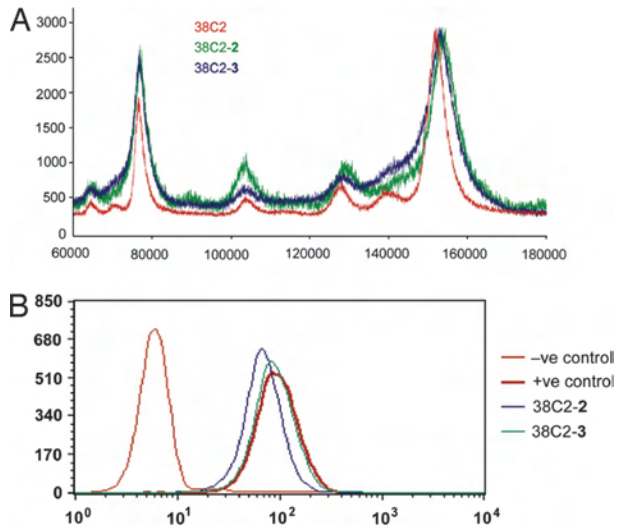


Fig. 2. Analysis of the catalytic Ab conjugates. (*A*) Comparison of 38C2-**2** ($M_{r\ avg}$ = 153,758) and 38C2-**3** ($M_{r\ avg}$ = 153,046) conjugates to untreated 38C2 ($M_{r\ avg}$ = 151,997) by MALDI-MS analysis. (*B*) Flow cytometry histogram showing the binding of 38C2 conjugates, 38C2-**2** and 38C2-**3**, to integrin $\alpha_v\beta_3$ expressing human breast cancer cell line MDA-MB-231. Conjugates 38C2-**2** and 38C2-**3**, 38C2 alone, and the previously described chemically programmed 38C2 (cp38C2) construct (17) obtained from a diketone derivative of 1 were used at 5 μg/ml concentration. In all experiments, FITC-conjugated goat anti-mouse secondary Abs were used for detection. The *y* axis gives the number of events in linear scale, and the *x* axis gives the fluorescence intensity in logarithmic scale.

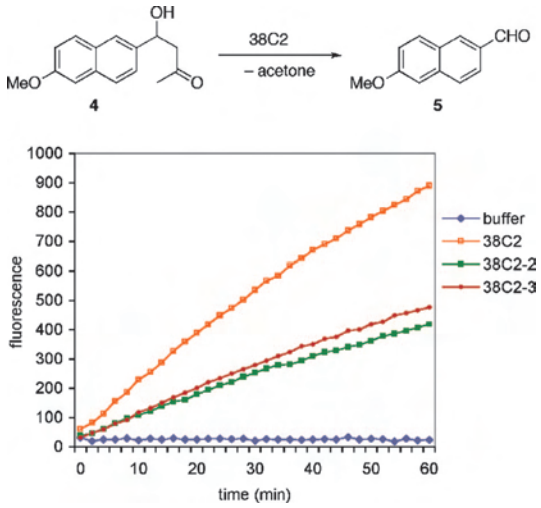


Fig. 3. Catalytic activity of 38C2-**2** and 38C2-**3** in comparison to free 38C2. Shown are rate of the *retro*-aldol reaction of aldol 4 to produce aldehyde 5 in the presence of the catalytic amounts of 38C2-**2**, 38C2-**3**, and free 38C2. The negative control reaction was in PBS buffer (50 mM).

it was necessary to determine that the conjugation chemistry did not destroy the catalytic activity of the Abs. To examine the catalytic activity of conjugates 38C2-**2** and 38C2-**3**, we used methodol **4** as a substrate, because it was known to undergo 38C2-catalyzed *retro*-aldol reaction to produce the fluorescent aldehyde **5**. Untreated 38C2 and PBS were used in the control experiments (20), and the progress of reaction was monitored by using a fluorescence reader (Fig. 3). As shown in Fig. 3, both 38C2-**2** and 38C2-**3** constructs retained ≈50% catalytic activity with respect to the untreated 38C2.

Synthesis of Prodox Partners for the Ab Catalysts. The next task was to develop prodrugs of dox that could be selectively activated by Ab 38C2. Earlier, several prodrugs of dox, **6**, including prodoxs **7-8** (Fig. 4*A*), were prepared and evaluated (6, 7, 21). On treatment with a catalytic amount of 38C2, all prodoxs were activated. Prodox **8** was activated faster than **7**, probably because of the longer linker. However, the background activation also increased with **8** because of the uncatalyzed hydrolysis of the aromatic carbamates. Obviously, a longer but stable linker was needed. Therefore, use of 4-aminobenzyl alcohol derived linkers as in prodoxs **9-12**, instead of the 4-hydroxymethyl-2-methoxyphenoxy-carbonyl-*N*,*N*′-dimethylethylenediamine of **8**, was examined (Fig. 4*B*). Conceivably, this functionality should reduce the background reaction of the aromatic carbamate but not compromise the rate of prodrug activation. Thus, the 38C2-catalyzed *retro*-aldol reaction of **9-12** and β elimination of the resultant intermediates, followed by decarboxylation, would afford intermediate **I**. Release of dox and 4-aminobenzylalcohol from intermediate **I** was known to be facile (22).

Alternatively, a new kind of aromatic-aldol linker could be conceived that would be obtained from 4-hydroxyacetophenone and connected to dox **6** through a carbamate functionality as in prodoxs **13-14** (Fig. 4*C*). Such an aldol linker would undergo the Ab 38C2-catalyzed activation at a much faster rate in comparison to an analogous aliphatic aldol linker, affording the electronically deficient ketone intermediate **II**. In fact, aldol compounds prepared from an aromatic ketone, such as 6-methoxy-2-methylcarbonyl naphthalene or 4-methoxyacetophenone, were among those substrates that were activated by 38C2 at >1 per min (2). The enhanced reactivity of the carbamate function in **II** would accelerate its hydrolysis to produce free dox. In the latter approach, however, one main concern was the noncatalyzed

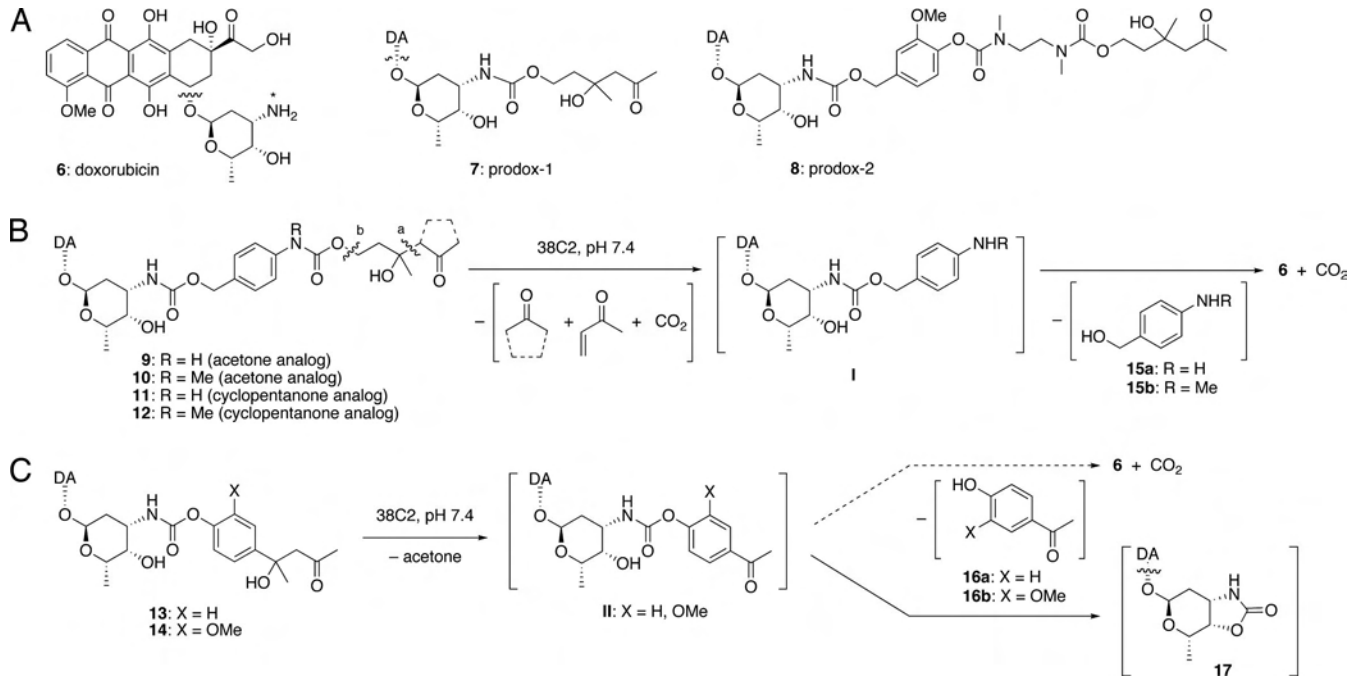
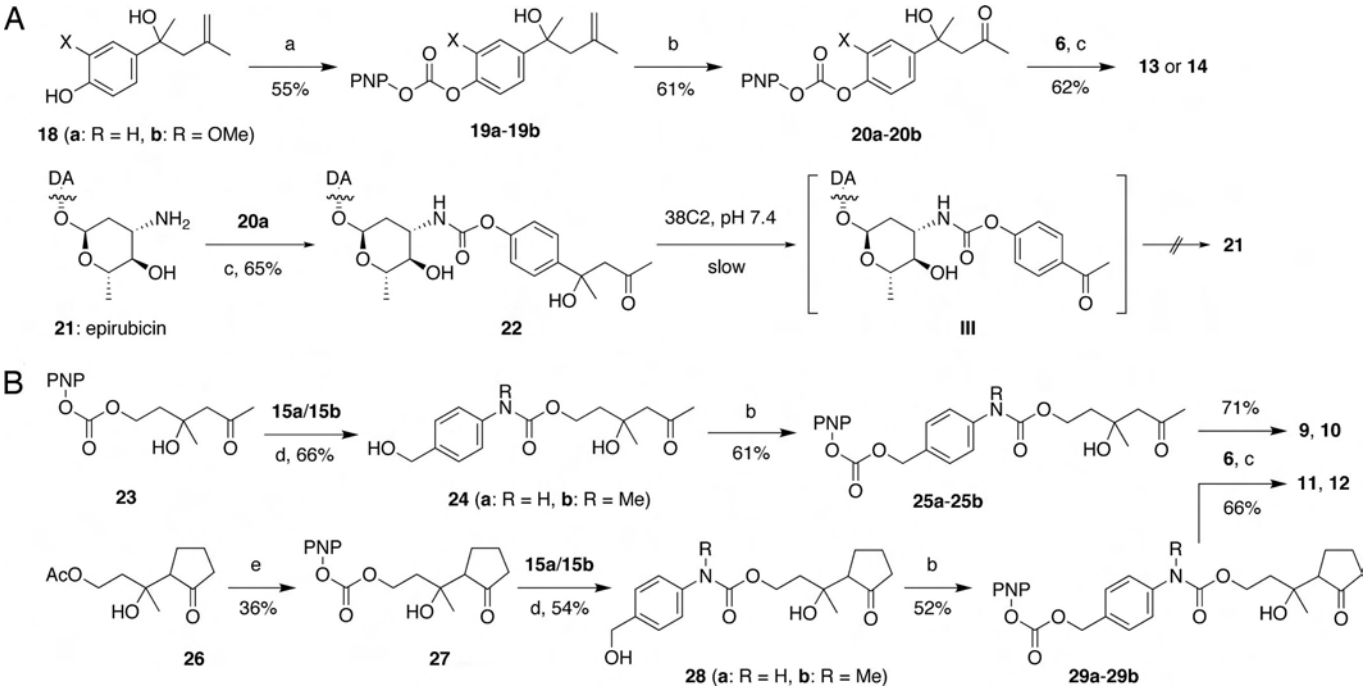


Fig. 4. Synthesis and activation of prodoxs. (*A*) Structure of dox and its prodrugs (prodoxs). (*B* and *C*) Structure of prodoxs and schemes showing 38C2-catalyzed activation of these prodoxs to produce dox via the labile intermediates. DA, dox aglycon.

hydrolysis of the aromatic carbamate. With all these considerations in mind, first we concentrated on the second set of prodrugs, **13-14**, which differ from each other only in the substituent on the phenyl ring. Here, the methoxyphenyl ring in **14** was introduced to modulate the background reactivity of the aromatic carbamate functions in the prodrugs.

Syntheses of prodoxs **13-14** were achieved by reacting dox hydrochloride with the aldol linkers **20a** and **20b** (Scheme 1*A*), which were prepared from phenols **18a-18b**, via **19a-19b** [see supporting information (SI)]. Next, we examined the activation of prodoxs **13-14** by using a catalytic amount of 38C2. As expected, activation of **13-14** to produce intermediate **II** was

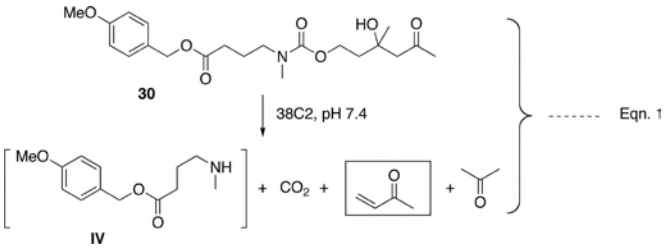


Scheme 1. Synthesis of prodoxs **13-14** and epirubicin prodrugs **22** (*A*) and prodoxs **9–12** (*B*). DA, dox aglycon; PNP, 4-nitrophenyl; RT, room temperature. Yields are given for compounds leading to **9**, **11**, and **13**. Comparable yields were obtained for their analogs. (a) 4-Nitrophenyl chloroformate, Py, CH_2Cl_2, 0°C; (b) (*i*) OsO_4 (cat), NMO, citric acid, CH_2Cl_2-H_2O (10:1), RT, (*ii*) $NaIO_4$, THF- H_2O (1:1), RT; (c) Et_3N, DMF; (d) DIPEA, HOBT, DMF, RT; (e) (*i*) DIBAL-H, CH_2Cl_2, −10°C, (*ii*) step a, and (*iii*) Dess Martin periodinane, CH_2Cl_2, RT.

quite fast; however, no dox was produced from these prodrugs. Instead, a compound **17** was produced from both **13-14** (Fig. 4*C*). Obviously, the intramolecular attack by the vicinal hydroxy function of prodox prevailed over an intermolecular attack by water. Considering that the hydroxy and amine or carbamate functions in dox or prodoxs are in *syn* positions, thereby accelerating the formation of **17**, we prepared an analogous prodrug **22** by using epirubicin, **21**. Notably, the hydroxy and amine or carbamate functions in **21** and **22** lie in anti position and should not form the cyclic carbamate. In this case, the 38C2-catalyzed conversion of the prodrug to the ketone intermediate **III** was slow, and no epirubicin was reproduced.

Because compounds **13-14** and **22** were not suitable for the prodrug therapy by using aldolase Abs, we focused on prodoxs **9-12**, in that prodoxs **11** and **12** were the cyclopentanone analogs of **9** and **10**, respectively. Because cyclopentanone was found to be a very efficient donor in the 38C2-catalyzed aldol reaction (23), it was anticipated that prodrugs **11-12** could undergo the *retro*-aldol reaction faster than **9-10**. The prodoxs **9-12** were synthesized by using dox hydrochloride and the aldol linkers, **25a-25b** and **29a-29b**, respectively (Scheme 1*B*). Syntheses of linkers **25a-25b** were achieved by alcohols **24a-24b** starting from **15a-15b** and the previously described nitrophenyl carbonate **23** (6). Linkers **29a-29b** were prepared from an aldol compound **26** that was prepared by Mukaiyama aldol reaction (24) of 4-acetoxy-2-butanone with 1-trimethylsilyloxypentene, by intermediates **27** and **28a-28b** (see SI).

We analyzed dox release from prodoxs **7** and **9-12** (100 μM solution) in the presence of a catalytic amount (1 μM solution) of Ab 38C2. Under identical conditions at 37°C, prodrugs **9-12** produced dox with an average rate of up to 50 times faster than the previously reported prodrug **7**. Thus, ≈10–30 times excess of dox were produced in 5 h from prodoxs **9-12**, respectively, as compared with that from prodox **7** over the identical time periods. Interestingly, however, complete consumption of either **7** or **9-12** was not seen, even when the reaction mixture was left at 37°C for an extended period. We anticipated that the critical lysine residues in the Ab 38C2-binding sites underwent conjugate addition to methyl vinyl ketone (MVK), which was produced during the prodrug activation, thereby inhibiting the catalytic activity of the Ab (25).‖ To test this, Ab 38C2 (1μM solution) was treated with a prodrug linker **30** (100 equivalent) that possessed the aldol–Michael motif (Eq. **1**) or with MVK (100 equivalent), and the activity of the mixtures was analyzed. Buffer alone was



used as the negative control.

The catalytic activities of the Ab mixtures and Ab and buffer alone were determined by using the conversion of aldol **4** (200 μM solution) to aldehyde **5**, as described above. Ab 38C2 was completely deactivated in <1 h when incubated with **30** or MVK (see SI). These observations were pertinent to our studies, because prodoxs **9-12** also produced MVK on activation with 38C2. However, whereas the production of MVK is a problem for studies *in vitro*, it should not be a problem *in vivo*, where it is expected to be rapidly cleared by cellular uptake and/or dilution into the large volume of total body fluids. This reasoning is supported by the fact that a solution containing 1 μM concentration of 38C2 and 10 equivalent of MVK required >24 h to achieve 90% inactivation of 38C2. An intriguing but as-yet-unproven possibility is that, as a Michael acceptor, any MVK taken up by the neoplastic cells could augment the cytotoxic effect of the activated prodrug, thereby affording combination therapy. Indeed, one can think of these constructs as triple therapy involving the prodrug, MVK, and the effector function of the Ab itself.

Tumor Cell Killing by Prodoxs 9–12 in the Presence and Absence of Abs. We evaluated toxicity of prodoxs **9-12** as compared with the previously described prodox **7** (6) and dox by using human breast cancer cells, MDA-MB-231. An analysis of the results showed that, under the described conditions, dox was toxic to cells at IC_{50} ≈ 5 μM (Fig. 5*A*). Prodoxs **10** and **12** were also quite toxic with an IC_{50} value of ≈20 μM for both of them. In contrast, prodoxs **9** and **11** did not show any appreciable toxicity up to 100 μM concentration (Fig. 5 *B* and *C*). Therefore, we used prodoxs **9** and **11** for further experiments.

Prodoxs **9** and **11** were compared with **7** in the presence and absence of Ab 38C2 in a cell proliferation assay by using MDA-MB-231 cells as described earlier (9). Both prodoxs **9** and **11** were shown to inhibit cell growth in a fashion similar to **7** in the presence of 1 μM Ab 38C2 concentration. The cytotoxicity profiles of **7**, **9**, and **11** in the presence of 38C2 were virtually identical to **6** (Fig. 5 *B* and *C*). The identical cytotoxicity profile of prodox **7** to **9** or **11** in the presence of 38C2, however, was in contrast to our earlier observation that **7** was activated slower than **9** and **11**, suggesting that the large amount of Ab used masked the differences in prodrug activation among the various analogues. This led us to speculate that Ab concentration could be further reduced in these experiments, without changing the cytotoxicity profile of prodoxs **9** and **11**. Indeed, when Ab concentration was reduced to 0.1 or 0.033 μM, differences among the various prodrugs became quite clear (Fig. 5*D*). These data suggested that prodoxs **9** or **11** would be better than **7** when studies *in vivo* are carried out.

To determine the efficacy for cell killing of the Ab conjugates that contained the targeting moiety, we compared 38C2 to the 38C2 conjugates 38C2-**2** and 38C2-**3** (Fig. 6). As evident from Fig. 6 *A* and *B*, prodox **11** showed the highest efficacy both in the presence of 38C2 and its constructs, 38C2-**2** or 38C2-**3**. The previously described prodox **7** was less efficient than both **9** and **11**. Therefore, of all of the prodoxs so far tested *in vitro*, **11** seems the best companion for the targeting Ab catalysts. Although we have not optimized the 38C2 concentration that will be required for the prodrug activation *in vivo*, it is evident from Fig. 5*D* that 38C2 could be used at even less than a concentration of 0.033 μM, because prodox **11** showed identical efficacy when 38C2 was used at 0.033- and 0.1-μM concentrations.

Conclusion

Ab conjugates were prepared by using Ab 38C2 and a small-molecule antagonist of integrin $\alpha_v\beta_3$. The conjugates bound efficiently to cells expressing integrin $\alpha_v\beta_3$ and catalyzed prodrug activation. In addition, a set of dox prodrugs with improved stability and lower toxicity was synthesized. *In vitro* evaluations using these Ab conjugates together with the dox prodrugs revealed that cell targeting and prodrug activation capabilities could be efficiently combined. We anticipate that prodox **11** and Ab conjugates, 38C2-**2** or 38C2-**3**, may be an appropriate com-

‖Inactivation of 38C2 by conjugate addition to MVK was evident from our other study (see ref. 17), as well as that from the Gouverneur laboratory showing no catalysis when vinyl ketones were used as substrates of the related Abs 84G3 and 93F3 (see ref. 25).

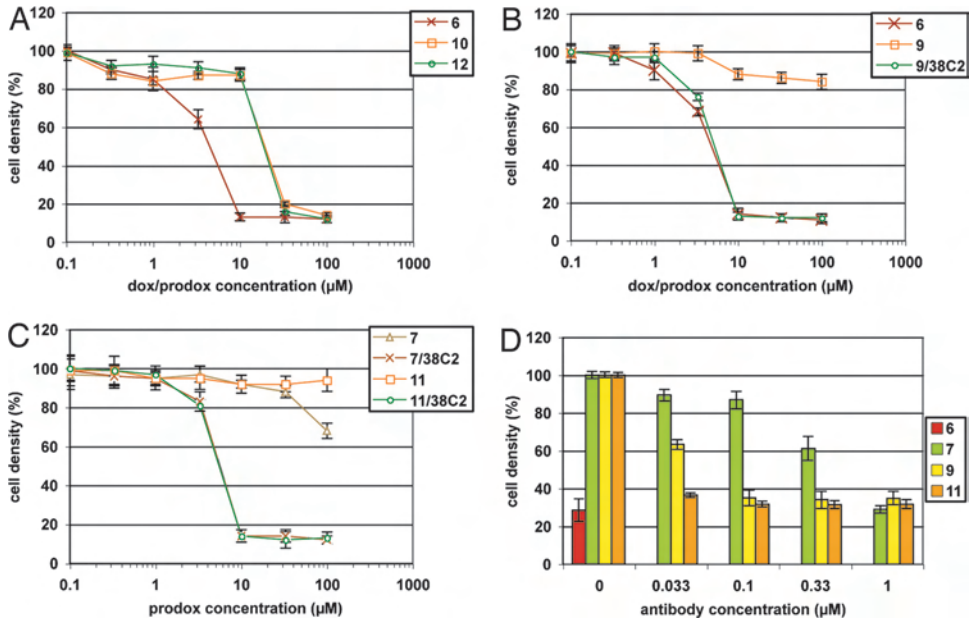


Fig. 5. Effect of dox and prodoxs 10 and 12 (*A*), dox and prodox 9 (*B*), prodoxs 7 and 11 (*C*), and dox and prodoxs 7, 9, and 11 (*D*) on human breast cancer cells, MDA-MB-231, *in vitro*, in the absence or presence of a catalytic amount of Ab 38C2. Ab 38C2 was used at a 1 μM concentration in *B* and *C*. Cells (20,000) were used and developed by using the Cell Titer 96 AQ_{ueous} One Solution Cell Proliferation Assay kit after 72 h of incubation with drug, prodrug, or prodrug/38C2 combination. In *D*, dox was used at a 10 μM concentration. The *y* axis shows cell density in a linear scale, and the *x* axis shows the dox or prodox concentration in a logarithmic scale in *A–C* and Ab concentration in a linear scale in *D*.

bination for *in vivo* use as antitumor and/or antiangiogenic therapeutic Abs.

Materials and Methods

Ab, Cell Lines, Reagents, and Prodrugs. The generation and purification of mouse Ab 38C2 have been described elsewhere (4). Human breast cancer cell line MDA-MB-231 was obtained from American Type Culture Collection, (Manassas, VA). The cells were cultured in Leibovitz L15 medium supplemented with 2 mM L-glutamine, and 10% FCS at 37°C in a CO_2-free environment. FITC-conjugated goat anti-mouse Ab was purchased from Chemicon, Temecula, CA. The Cell Titer 96 AQ_{ueous} One Solution Cell Proliferation Assay kit was purchased from Promega, Madison, WI. Syntheses of prodrugs are described in the SI.

Preparation of the Integrin $\alpha_v\beta_3$-Targeting Ab 38C2 Conjugates. The preparation of 38C2-**2** conjugate is as follows: Ab 38C2 (1 mg/ml, 3 ml) in PBS buffer (pH 7.4) was reduced by using DTT solution (0.14 μmol) at 37°C for 3 h under argon. The solution was dialyzed by using PBS buffer, pH 6.0, under argon. To this solution, compound **2** (0.18 mg/0.2 μmol in 10 μl of dimethylformamide) was added, and the mixture was left at 4°C for 16 h. The reaction mixture was dialyzed by using PBS buffer (pH 7.4) to afford the 38C2-**2** conjugate.

The preparation of the 38C2-**3** conjugate was as follows: a solution of pentane-2,4-dione (2 μl of 100 mM solution in CH_3CN) was added to 38C2 (1 mg/ml, 3 ml) in PBS buffer (pH 7.4) at room temperature to temporarily block the reactive lysine residues in the Ab 38C2-binding sites. After mixing the solution for 2 h, a solution of **3** (0.19 mg in 50 μl of CH_3CN) was added, and the mixture was left at room temperature with continuous mixing for 16 h. The resultant 38C2-**3** conjugate was reactivated by dialyzing the mixture using PBS (pH 7.4) containing hydrazine (1%), and then using PBS (pH, 7.4) alone.

Evaluation of the Binding of 38C2 Conjugates to Integrin $\alpha_v\beta_3$-Expressing Cells. Binding of 38C2-**2** and 38C2-**3** was evaluated by using integrin $\alpha_v\beta_3$-expressing cells, as described (17). Briefly, aliquots of 100 μl containing 1×10^5 cells were distributed into wells of a V-bottom 96-well plate for indirect immunofluores-

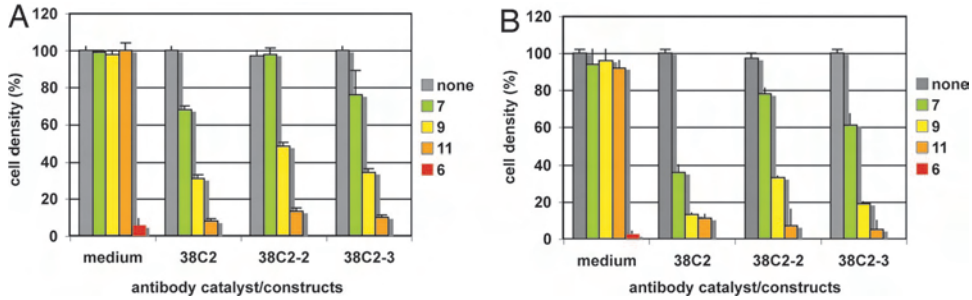


Fig. 6. Effect of dox and prodoxs **7**, **9**, and **11** on human breast cancer cells, MDA-MB-231, *in vitro*, in the absence and/or presence of 38C2 (0.1 μM concentration) or 38C2 conjugates (0.2 μM concentration). Dox and prodoxs were used at 5 μM (*A*) and 10 μM (*B*) concentrations. The experiments were conducted as described earlier, except that cells were used at a lower number (3,000 cells per well) and developed after 120 h of incubation with dox, prodox/38C2, prodox/38C2-**2**, or prodox/38C2-**3** combinations. The *y* axis shows cell density in a linear scale, and the *x* axis shows the buffer or catalyst used.

cence staining. After centrifugation for 2 min, they were resuspended by using 100 μl of the Ab conjugates (38C2-**2** and 38C2-**3**), Ab 38C2 alone, and the previously described chemically programmed 38C2 construct (5 μg/ml cp38C2) in flow cytometry buffer. After incubating for 1 h, the complex samples were centrifuged, washed twice, and resuspended by using 100 μl of a 10 μg/ml solution of FITC-conjugated goat anti-mouse polyclonal Abs in flow cytometry buffer. After these samples were further incubated for 45 min at room temperature, flow cytometry was performed by using a FACScan instrument.

Evaluation of the Catalytic Activities of 38C2 Conjugates. A solution of 38C2 and its conjugates, 38C2-**2** and 38C2-**3** (98 μl of 0.6 μM solution in PBS) and buffer alone (98 μl) were transferred in four different wells of a 96-well fluorescence measuring plate. Methodol (**4;** 2 μl of 10 mM solution in CH_3CN) was added to the Ab, Ab conjugates, and buffer-containing wells, and the rate of formation of 6-methoxynaphthaldehyde, **5**, was determined by using a fluorescence reader.

Evaluation of the Prodrug-Mediated Cellular Toxicity in the Absence and Presence of 38C2 Conjugates. Stock solutions (10 mM) of dox **6** and prodoxs **7** and **9**–**12** were prepared in DMSO and stored at 4°C. The cell growth assay was carried out by using MDA-MB-231 human breast cancer cells (obtained from American Type Culture Collection). Briefly, cells were plated at a density of 20,000 and 3,000 (used for experiments shown in Figs. 5 and 6, respectively) per well in 96-well tissue culture plates and maintained in culture. Prodrugs were added to the cells 24 h after plating, making 0.01 to 100 μM final concentrations for the prodrugs. For the Ab experiments, prodox and 38C2 or 32C2 conjugates were mixed just before adding to the cells. After prodox addition, the cells were maintained at 37°C in 5% CO_2 for 72 or 120 h and developed by using the Cell Titer 96 AQ_{ueous} One Solution Cell Proliferation Assay kit. Results are depicted in Fig. 5. In experiment (Figs. 5*D* and 6*B*), dox and prodoxs were used at a 10 μM concentration, and in Fig. 6*A*, they were used at 5 μM concentration.

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